INVESTIGATION OF CLONALITY AND MINIMAL RESIDUAL DISEASE IN HAEMATOLOGICAL MALIGNANCY USING FLUORESCENT IN SITU HYBRIDIZATION

A thesis submitted for the degree of Doctor of Philosophy at the University of London

by Arkadiusz Kasprzyk

Haematology Department, The Royal Free Hospital Medical School, London NW3 2QG

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ABSTRACT

Cytogenetic analysis of the malignant clone is clinically important in haematological malignancy. Analysis by metaphase cytogenetics is restricted to the small proportion of malignant cells which are actively dividing. This thesis explores the dynamics of malignant clones using the technique of fluorescence *in situ* hybridization (FISH) to visualize chromosomal abnormalities in interphase (non-dividing) cells. Hyperdiploid (>46 chromosomes) clones have been investigated by interphase FISH in acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS) using appropriate chromosome-specific probes. A hyperdiploid clone was detected in interphase cells in 9/65 patients with ALL in whom metaphase cytogenetics had failed or was normal. A single hyperdiploid cell was identified as clonal in one patient with MDS but not in six others with AML, MDS or ALL. The involvement of different cell lineages in the malignant clone was investigated by simultaneous FISH and identification of the cell type by morphology or monoclonal antibodies. In ALL, hyperdiploid clones were restricted to the lymphoid blasts in 9/9 cases, while Philadelphia (Ph) positive clones, (identified by probes to the genes m-*BCR* or M-*BCR* and *ABL* which fuse as a result of the translocation) were found either in lymphoid blasts alone (1/3 cases) or in both lymphoid and myeloid cells (2/3 cases). In AML trisomy 8 (using a chromosome 8-specific probe) and an 11q23 abnormality (which split YAC 13HH4) were both found only in the myeloid blasts, in 3/3 and 2/2 cases respectively. A sensitive method for the detection of hyperdiploid ≥50 clones in ALL was developed for minimal residual disease detection. Simultaneous probing of three chromosomes enabled detection of one hyperdiploid cell in 10,000. Heterogeneity in the speed with which the clone was eliminated in remission was seen in 16 patients and early relapse was detected in one patient.

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DECLARATION

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other University or Institute of Learning.

Arkadiusz Kasprzyk

ABBREVIATIONS

ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloblastic Leukaemia
APAAP	Alkaline Phosphatase Anti-Alkaline Phosphatase
BMT	Bone Marrow Transplant
CD	Cluster of Differentiation
CSF	Colony Stimulating Factor
CFU-E	Colony Forming Unit Erythroid
CFU-Eo	Colony Forming Unit Eosinophil
CFU-G	Colony Forming Unit Granulocyte
CFU-GEMM	Colony Forming Unit Granulocyte, Erythroid , Monocyte, Megakaryoblast
CFU-GM	Colony Forming Unit Granulocyte, Monocyte
CFU-Mk	Colony Forming Unit Megakaryoblast
CNS	Central Nervous System
DAPI	4', 6-Diamidino-2-Phenylindole
FAB	French-American-British
FISH	Fluorescence *In Situ* Hybridization
FITC	Fluorescein Isothiocyanate
G6PD	Glucose-6-Phosphatase
HD	High Density fraction
HGF	Haematopoietic Growth Factors
HM	Haemopoietic Microenviroment
Ig	Immunoglobulin
IL	Interleukin
MAC	Morphology, Antibody, Chromosome
Mb	Megabase
MDS	Myelodysplastic Syndrome
MGG	May Grunwald Giemsa staining
MNC	Mononuclear Cells
MPD	Myeloproliferative Disorder
MRD	Minimal Residual Disease
Ph	The Philadelphia Chromosome

PI	Propidium Iodide
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
PV	Polycythemia Vera
RA	Refractory Anaemia
RAEB	Refractory Anaemia with Excess of Blasts
RAEBT	Refractory Anaemia with Excess of Blasts in Transformation
RAS	Refractory Anemia with Sideroblasts
RFLP	Restriction Fragment Length Polymorphism
RCLB	Red Cell Lysis Buffer
RT-PCR	Reverse Transcryptase Polymerase Chain Reaction
SSC	Standard Saline Citrate
TBS	Tris Buffered Saline
TCR	T-Cell Receptor
WCP	Whole Chromosome Probe
WBC	White Blood Cell count
YAC	Yeast Artificial Chromosome

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INTRODUCTION

1.1 CYTOGENETICS AND FLUORESCENT *IN SITU* HYBRIDIZATION IN THE STUDY OF HAEMATOLOGICAL MALIGNANCY

Cytogenetics has established itself as an important tool in haematological practice. A number of chromosomal abnormalities have been recognized that are very closely associated with subtypes of leukaemia and lymphoma. The detection of one of these recurring abnormalities can be quite helpful in establishing the correct diagnosis and can add information of prognostic importance.

The application of fluorescence *in situ* hybridization (FISH) in the study of haematological malignancy has expanded rapidly since its invention in 1969 and the technique is now widely used in haematology as the means of supplementing cytogenetics both for diagnostic purposes and in research.

1.1.1 Cytogenetics

The clinical importance of chromosomal abnormalities has been demonstrated in acute lymphoblastic leukaemia (ALL), in acute myeloblastic leukaemia (AML) and in myelodysplastic syndrome (MDS). In the following section, the important features of each of the above diseases is mentioned briefly and the clinical importance of the major non-random chromosomal abnormalities is discussed.

1.1.1.1 Acute Lymphoblastic Leukaemia (ALL)

Acute lymphoblastic leukaemia is characterized by the accumulation of malignant, immature lymphoid cells in the bone marrow and often in peripheral blood. The disease is much more common in children than in adults. The incidence in childhood is 3 per 100 000. Males are more often affected than females.

1.1.1.1.1 FAB classification

The classification of ALL by the morphology of the blasts was devised by a French, American and British consortium and is known as the FAB classification (Bennett et al., 1976; Bennett et al., 1982; Bennett et al., 1985). This has been used as a means of distinguishing three subtypes of ALL. L1 describes ALL with blasts which are mainly small and relatively uniform in appearance with nuclei of regular shape and

indistinct nucleoi. L2 describes ALL with blasts that are larger than L1 and more heterogeneous. The nuclear shape is more irregular and one or more nucleoi may be present. L3 describes ALL with blasts which are large and homogeneous. Prominent vacuolation of the basophilic cytoplasm is often present (Bain, 1998b).

1.1.1.1.2 Immunological classification

The use of monoclonal antibodies for the identification of cell surface antigens has helped us to understand the stages of differentiation of leukaemia and has provided means to further subdivide ALL. Thus, ALL is divided into two major categories; B and T-lineage ALL. There are further subdivisions within each category according to the expression of antigens known as clusters of differentiation (CD) which is related to the degree of the maturation of blast cell.

B-lineage

B lineage ALL is defined by the expression of at least two of the following three early B cell markers: CD19, CD79 or CD22. The major immunological classes are as follows: pro-B-ALL (B-I) with no expression of other differentiation B-cell antigens, common-ALL (B-II) which is CD10 +ve, pre-B-ALL (B-III) which shows intracytoplasmic μ chains and mature B-ALL (B-IV) which shows cytoplasmic or surface kappa or lambda chains (European Group for the Immunological Characterization of Leukemias (EGIL) et al., 1995).

T-lineage

T-lineage ALL is defined by the cytoplasmic or membrane expression of CD3. Four subgroups of T-ALL are defined as follows: pro-T-ALL (T-I) which is CD7 +ve, pre-T-ALL (T-II) which is CD2 +ve and/or CD5 +ve and/or CD8 +ve, cortical-T-ALL (T-III) which is CD1a +ve and mature-T-ALL (T-IV) which is membrane CD3 +ve and CD1a -ve (European Group for the Immunological Characterization of Leukemias (EGIL) et al., 1995).

1.1.1.1.3 Chromosomal abnormalities

An abnormal karyotype can now be demonstrated in between 44% and 90% of cases (IWCL3, 1981a; Williams et al., 1985; Fenaux et al., 1989; Secker-Walker et al., 1989; Crist et al., 1990; Jackson et al., 1990; Pui et al., 1990; Secker-Walker, 1990; Walters et al., 1990; Secker-Walker et al., 1991; Dastugue et al., 1992; Van der Plas

et al., 1992a; GFCH, 1993). ALL can be classified by numerical or structural cytogenetic features.

Structural change

t(1;19) (q23;p13) and der(19)t(1;19)

This translocation is found in between 2% and 9% of ALL (GFCH, 1993; Harbott et al., 1993; Pui et al., 1994). It is strongly associated with a pre-B immunophenotype. The translocation can occur as a balanced t(1;19)(q23;p13) or an unbalanced variant der(1;19)t(1;19). At the molecular level the translocation results in the juxtaposition of the genes *E2A* (chromosome 19) and *PBX1* (chromosome 1). The prognostic significance of this abnormality is unclear. The association with poor risk features was shown in some reports (Lai et al., 1989). Other researchers failed to show any such correlation (Carroll et al., 1984; Michael et al., 1984). The intensification of treatment regimens appear to improve prognosis. Also the unbalanced variant of the translocation was shown to be associated with better prognosis than its balanced counterpart (Secker-Walker et al., 1992a).

t(4;11)(q21;q23)

The incidence of this translocation has been reported as 2% in children (Mirro et al., 1986; Secker-Walker, 1990) and 5% in adults (IWCL3, 1981a). However, it is now clear that most patients with t(4;11) are infants (<1 years old). The incidence in this group of patients has been shown to be between 30% and 70% (Lampert et al., 1992; Ferster et al., 1994; Heerema et al., 1994). The t(4;11) is associated with a null-cell immunophenotype (CD19+, CD10-) (Pui, 1992) and high (>100x10^9/l) WBC. The t(4;11) has been associated with myeloid characteristics, which indicates possible pluripotent stem cell involvement (IWCL3, 1981a; Morse et al., 1982; Parkin et al., 1982; Nagasaka et al., 1983; Levin et al., 1984; Stong et al., 1985). It has been shown that at the molecular level the abnormalities involving 11q23 often lead to rearrangement of the *MLL (ALL1, HRX, HTRX1)* gene (Cimino et al., 1991; Ziemin-van der Poel et al., 1991; Das et al., 1992; Djabali et al., 1992; Tkachuk et al., 1992; Thirman et al., 1993) and that the t(4;11) results in a fusion between the *MLL* (chromosome 11) and the *AF4 (FEL, LTG4, MLLT2)* gene on chromosome 4 (Gu et al., 1992; Chen et al., 1993; Corral et al., 1993; Domer et al., 1993; Morrissey et al., 1993). Patients with this translocation have a poorer prognosis than that of any other

chromosomal subgroup. The average survival is probably no more than 6 months (IWCL3, 1981a; Secker-Walker, 1990), but longer survival have been amongst 2-10 year olds (Johansson et al., 1998).

t(9;22)(q34;q11)

The Philadelphia chromosome which is one of the products of the t(9;22), was first described by Nowell and Hungerford in 1960 (Nowell, Hungerford, 1960). It occurs in more than 90% of CMLs and identifies a prognostically important subgroup of patients with ALL. The incidence of t(9;22) in childhood is between 2.3% and 5% (Secker-Walker et al., 1976; Crist et al., 1990; Secker-Walker, 1990; Fletcher et al., 1992). In adult ALL the Philadelphia chromosome occurs in 11% to 17% of cases (IWCL3, 1981b; Secker-Walker et al., 1997).

The Ph translocation has been associated with a high (>$30x10^9$/l) white blood cell count (WBC). The difference between WBC in Ph+ ALL and Ph- ALL is particularly pronounced in childhood ALL (Crist et al., 1990). Blast cell immunophenotype has been shown to be mostly c-ALL (74%), followed by pre-B (16%) and T-ALL (10%) (Ribeiro et al., 1987; Crist et al., 1990).

The Philadelphia translocation results in the fusion of part of the *ABL* gene, located on chromosome 9 and the *BCR* gene, located on chromosome 22. There are two different breakpoint cluster regions within the *BCR* gene: the 5.8 kb major breakpoint cluster (M-*BCR*) region and the 35 kb minor cluster region (m-*BCR*). Fusion between M-*BCR* and *ABL* gives rise to p210 protein while fusion between m-*BCR* and *ABL* gives rise to p190 protein. M-*BCR* is the breakpoint in 99% of Ph positive CML cases and in 50% of Ph positive ALL cases. The remaining 50% of Ph+ ALL have a m-*BCR* breakpoint. Prognosis for patients with ALL and this translocation is poor. In childhood, the remission rate of Ph+ ALL is 78% to 80% as compared with 90%-99% in other ALL cases (Crist et al., 1990; Fletcher et al., 1992) and median event-free survival is only 12-18 months. The event free survival in childhood ALL by five years is no more than 15% (Ribeiro et al., 1987; GFCH, 1993; Harbott et al., 1993). Similarly in adult ALL, patients with this translocation do worse than those without (Bloomfield et al., 1989; Secker-Walker et al., 1991; Gotz et al., 1992a). The median event free survival in adult Ph+ ALL at three years is less than 14% (Gotz et al., 1992b; Secker-Walker et al., 1997).

The Ph translocation is accompanied by additional abnormalities at diagnosis in up to 60% of children and 90% of adults with ALL (IWCL3, 1981b; Gotz et al., 1992b). In general this appears to be unimportant in terms of prognosis but Ph+ ALL with monosomy 7 has a particularly poor prognosis (Russo et al., 1991).

t(8;14)(q24;q32), t(2,8)(p12;q24) and t(8,22)(q24;q11)

The three translocations are found in mature B-cell ALL which is associated with FAB L3-Burkitt-type morphology. At least one of the above translocations is found in over 80% percent of Burkitt Lymphoma (BL) or ALL L3 cases (Kornblau et al., 1991). At the molecular level these three translocation share a common breakpoint at 8q24, the site of the oncogene *MYC*. The second breakpoint is at the site of an immunoglobulin chain gene, either the *IGK* light chain kappa at the 2p12, the *IGL* lambda at 22q11 or the heavy chain *IGH* at 14q32. Prognosis for this group using standard ALL therapy used to be extremely poor with median survival of lees than one year (IWCL3, 1981a). More recently the availability of special protocols, has improved survival of these cases.

t(1;14)(q32;q11) and t(8;14)(q24;q11)

These translocations are found in T-cell ALL. The t(1;14) is found in between 3% and 6% of T-ALL cases (Carroll et al., 1990; Chan et al., 1992). At the molecular level the translocations share a common breakpoint at 14q11, at the site of the T-cell receptor genes *TCRA*. In the t(1;14) the other gene involved is *TAL-1*, while in the t(8,14) the gene involved is the oncogene *MYC*. Prognosis for the patients with these translocation is poor and in the case of t(1;14) the median event-free survival is 4 months (Lange et al., 1992).

del 6q

Deletion of 6q is found in T and early B-lineage ALL and is found in 8% to 13% of childhood cases (IWCL3, 1981a; Crist et al., 1990; Dastugue et al., 1992) and 4% of adults (Secker-Walker et al., 1997). It involves breakpoints between 6q13-6q21 and 6q21-6q23.

del 9p

Deletion of 9q is found in T and early B-lineage ALL The incidence of del 9p occurs in between 5% and 10% of cases with T-ALL (Raimondi et al., 1988; Berger et al., 1990; Secker-Walker et al., 1992b; Van der Plas et al., 1992b; GFCH, 1993). At the molecular level the deletion is associated with the loss of the interferon genes *INFA*,

INFB and the multiple tumor suppresser gene *MTS1* and *MTS2*. This abnormality has been associated with 'lymphomatous disease': lymphadenopathy, splenomegaly, CNS involvement and high leukocyte count (Kowalczyk et al., 1985).

del 12p

The abnormalities of 12p include deletions and balanced and unbalanced translocations. The incidence of an 12p abnormality in children, by cytogenetics using banding techniques, has been shown to be 10% (Raimondi et al., 1986). The 12p translocations have proved to be very difficult to detect by G-banding due to pale staining of this region. It is now becoming clear that a proportion of the 12p deletions may now be reclassified as translocations. In 1994 Romana et al. investigated 8 patients with del(12p) using FISH with chromosome paints and showed that three of them had in fact t(12;21) a translocation that had not been previously described (Romana et al., 1994). At the molecular level the t(12;21) translocation results in the fusion of the *AML1* gene located at 21q22 and the *TEL* gene located at 12p13. Fusion of *TEL-AML1* has been seen by RT-PCR in up to 30% of children with ALL and it is associated with a very good prognosis. The projected event-free survival has been shown to be 74% at 3 years (Romana et al., 1995).

Numerical change

Hypodiploidy

Hypodiploidy is defined as a clonal loss of at least one chromosome. In ALL it occurs in between 5% and 8% of childhood patients (Minden et al., 1978; Secker-Walker et al., 1985; Pui et al., 1987; Prigogina et al., 1988; Crist et al., 1990; Jackson et al., 1990; Pui et al., 1990; Secker-Walker, 1990; Van der Plas et al., 1992a; GFCH, 1993) and in 9% of adult patients (Secker-Walker et al., 1990). There is no consensus with regard to the prognosis for those cases. In some surveys hypodiploid cases have shown a better prognosis than average (Secker-Walker et al., 1982a; Van der Plas et al., 1992a) in others they have had a worse prognosis (Bloomfield et al., 1989).

Near haploidy

Near haploidy is defined as a chromosomal gain from the haploid number of 23 chromosomes. The chromosomes frequently gained are 10, 14, 18, 21. Both sex chromosomes are frequently present. The incidence of near haploidy is in between

0.7% to 2.4% (Pui et al., 1987; Gibbons et al., 1991). The prognosis for cases with near haploidy is poor.

Pseudodiploidy

Pseudodiploidy is defined as the presence of 46 chromosomes and a structural change or loss of 1 or more chromosomes complemented by gain of the same number of other chromosomes. Pseudodiploidy accounts for 40% of cases with ALL (Crist et al., 1990; Jackson et al., 1990; Pui et al., 1990; Secker-Walker, 1990; Dastugue et al., 1992; GFCH, 1993). If hierarchical classification, in which structural change takes precedence over ploidy, is applied 10% of cases with this abnormality is found (Secker-Walker et al., 1997).

Low hyperdiploidy

Low hyperdiploidy is defined as the presence of 47-50 chromosomes. The chromosomes most frequently gained are 21, X, 8 and 10. The incidence of low hyperdiploidy in childhood ALL ranges from 11% to 15% (Jackson et al., 1990; Pui et al., 1990; Secker-Walker, 1990; Raimondi et al., 1992; GFCH, 1993). In adults low hyperdiploidy accounts for between 8% and 11% (Secker-Walker et al., 1990; Walters et al., 1990). There is little consistency in the prognosis for the patients with this abnormality. Both better (Bloomfield et al., 1989) and worse (IWCL3, 1981a) than average prognoses have been reported.

High hyperdiploidy

High hyperdiploidy is defined as the presence of 51-65 chromosomes. The chromosomes most frequently gained are X, 4, 6, 10, 14, 17, 18 and 21. The incidence of high hyperdiploidy in childhood ALL ranges from 16% to 27% (Pui et al., 1990; Secker-Walker, 1990; Dastugue et al., 1992; Van der Plas et al., 1992a; GFCH, 1993). In adults the incidence of high hyperdiploidy is remarkably lower and accounts for between 4% and 5% of the diagnostic cases (Pui et al., 1989; Secker-Walker et al., 1990; Walters et al., 1990). This group is associated with good risk factors: age 2-9 years, leukocyte counts less than $10x10^9/l$, female sex, C-ALL immunophenotype, FAB type L1. Patients with high hyperdiploidy enjoy the best prognosis. An average event-free survival of 5 years is achieved in 72% of children (Pui et al., 1989; Jackson et al., 1990; Van der Plas et al., 1992b).

An initial report that the presence of a structural change in addition to high hyperdiploidy has an adverse impact on prognosis (Pui et al., 1989) has not been

confirmed in a more recent report from the same group (Raimondi et al., 1996). The gain of a particular chromosome has been related to prognosis. Jackson et al. showed that trisomy 6 was associated with a good prognosis in patients with hyperdiploidy (Jackson et al., 1990). Harris et al. showed that combined trisomy of chromosomes 4 and 10 improves prognosis in patients with high hyperdiploidy (Harris et al., 1992). Hyperdiploid clones having a DNA index of more than 1.16 have been associated with a good prognosis (Look et al., 1985). Attempts have been made to uncover the mechanisms underlying the better prognosis of patients with high hyperdiploidy. The potential role of methotrexate, a basic component of treatment regimes in ALL, has been implicated in one report. High hyperdiploid cells have been shown to accumulate high levels of methothrexate and its metabolite, methothrexate polyglutamate, in vitro (Whitehead et al., 1992). The role of inferior survival capabilities of high hyperdiploid blasts in culture was shown in one report. Manabe et al. described a serum -free assay to compare survival requirements of leukaemic B-cell progenitors. In this assay the only two cases not giving rise to viable cells in culture were patients with a high hyperdiploid clone (Manabe et al., 1992).

Triploidy/Tetraploidy

Triploidy/Tetraploidy is defined as the presence of more than 65 chromosomes. It accounts for 7% of adults (Secker-Walker, 1990; Secker-Walker et al., 1990) but only 2.1% of children (Secker-Walker, 1998). The prognosis in adults is good and in children is unclear.

1.1.1.2 Acute Myeloid Leukaemia (AML)

Acute myeloid leukaemia describes malignancies of adults and children affecting non-lymphoid cells. In contrast to ALL it affects mostly adults and occurs mainly between the ages 22-60 years. The total incidence of AML is 2.5 cases per 100 000. The cell involved is an immature blast cell and disease is classified according to the features of this cell.

1.1.1.2.1 FAB classification

The FAB system classification is based on blast cell morphology. It includes 8 categories M0 to M7. Thus, M0 describes myeloblastic AML with minimal evidence of differentiation, M1 is myeloblastic without maturation, M2 is myeloblastic with

maturation, M3 promyelocytic, M4 myelomonocytic, M5 monoblastic, M6 erythroblastic and M7 megakaryoblastic (Bain, 1998b).

1.1.1.2.2 Chromosomal abnormalities

An abnormal karyotype can now be demonstrated in 69% to 84% of patients (Misawa et al., 1986; Schiffer et al., 1989; Rowley, 1990). The abnormal karyotype in AML involves numerical and/or structural changes.

Numerical change

Trisomy 8

This abnormality is the most frequent abnormality in AML and is seen in 13% of cases (IWCL4, 1984). This abnormality appears to be relatively specific to myeloid disorders. Trisomy 8 is not restricted to any particular FAB type but it is particularly frequent in M2, M4 and M5.

Monosomy 7

This is the second most frequent numerical abnormality in AML and is observed in 9% of cases (IWCL4, 1984)

Structural change

t(8;21)(q22;q22)

This translocation was first described by Rowley et al. in 1973 (Rowley, 1973). It is the most frequent structural abnormality in AML and it occurs in 15% of cases (Mitelman, Heim, 1992). It is also the most frequent abnormality seen in children with AML (Raimondi et al., 1989; Petkovic et al., 1992). This abnormality is strongly associated with the M2 variant of AML. Patients with this abnormality have a relatively favorable prognosis.

t(15;17)(q22;q11-21)

This abnormality was first described by Rowley et al (Rowley et al., 1977). This abnormality is highly specific to M3 variant of AML and has not been recognized in any other type of leukaemia. Patients with AML M3 are younger than other patients with AML (Larson et al., 1983; IWCL4, 1984; Larson et al., 1984). At the molecular level the translocation follows a break within the second intron of the retinoic acid receptor alpha gene (*RARA*) which is a part of the steroid/thyroid hormone receptor

superfamily. The truncated gene is then moved to chromosome 15 where it is fused to a gene called *PML* to give rise to a new *PML/RARA* hybrid gene. The reciprocal product of *PML/RARA* is found on chromosome 17.

inv(16)(p13;q22)

This rearrangement of chromosome 16 is detected in approximately 2% of the cases with AML. It has been strongly associated with AML M4 with eosinophilia (Arthur, Bloomfield, 1983). At the molecular level the inversion interrupts a myosin or smooth muscle heavy gene, *MYH 11* in 16p13 and in 16q22, the gene *CBFB* which normally encodes a subunit of a heterodimeric transcription factor. The subsequent inversion results in fusion of *MYH 11* and *CBFB* to create a chimeric gene

Rearrangements of 11q23

A breakpoint at 11q23 occurs in a number of translocations. The other chromosome involved most frequently is chromosome 9 as t(9;11)(p21;q23) (Hagemeijer et al., 1982), also seen is 6 as t(6;11)(q27;q23) (Martineau et al., 1998), 10 as t(10;11)(p11-p15;q23) (Berger et al., 1982; Kaneko et al., 1986), 17 as t(11;17)(q23;q25) (Berger et al., 1982; Dewald et al., 1983) and 19 as t(11;19)(q23;p13) (Berger et al., 1982; Vermaelen et al., 1983; Kaneko et al., 1986). The 11q23 breakpoint has been strongly associated with AML M5. In this subtype of AML the 11q23 abnormalities were found in 35% of patients (Berger et al., 1982). The association between 11q23 abnormalities and M5 is particularly strong in children (Rowley, 1983). The 11q23 rearrangements have been associated with lymphoid markers expression in AML (Cuneo et al., 1993; Drexler et al., 1993).

1.1.1.3 Myelodysplastic Syndrome (MDS)

Myelodysplastic syndrome constitutes a group of diseases of progressive bone marrow failure of normal maturation which leads to peripheral cytopenias. Usually more than one lineage is involved, very often three. It affects mostly elderly patients and the incidence is estimated as 1 per 100 000. Approximately 20% to 40% patients with MDS eventually develop AML.

1.1.1.3.1 FAB classification

This classification subdivides MDS on the basis of the percentage blasts and some morphological features. Thus, refractory anaemia (RA) describes MDS with less than

5% blasts and less than 15% ringed sideroblasts in nucleated red cells, refractory anemia with sideroblasts (RAS) describes an MDS variant with less than 5% blasts but more than 15% ringed sideroblasts in nucleated red cells, refractory anaemia with excess of blasts (RAEB) describes MDS with 5%-20% of blasts and finally refractory anaemia with excess of blasts in transformation (RAEBT) describes MDS with 21% to 29% of blasts (Bain, 1998a).

1.1.1.3.2 Chromosomal abnormalities

An abnormal karyotype can be demonstrated in 35% to 75% of patients with a primary myelodysplastic syndrome (Knapp et al., 1985; Jacobs et al., 1986; Kerkhofs et al., 1987; Billstrom et al., 1988; Horiike et al., 1988; Musilova, Michalova, 1988; Yunis et al., 1988; Pierre et al., 1989; Geddes et al., 1990; Jotterand-Bellomo et al., 1990; Suciu et al., 1990; Vila et al., 1990; Ohyashiki et al., 1991). In a review of 3000 cases with primary MDS, the most frequent chromosomal change was 5q- which accounted for 27% of all cases with chromosomal abnormalities (Mufti, 1992). This was followed by trisomy 8 (19%), monosomy 7 (15%), 11q- (7%), trisomy 7(5%), 12q- (5%), 20q- (5%), i(17q) (5%), 7q- (4%), t(1;7) (2%) and 13q- (2%). Rare abnormalities, accounting for 1% of cases, were as follows t(1;3), inv (3)(q21;q26) and t(3;3)(q21;q26).

5q- chromosome

The deletion of the long arm of chromosome 5, (5q-) constitutes 27% of all abnormal karyotypes in primary myelodysplastic syndrome (Mufti, 1992). When 5q- is associated with a combination symptoms, the presence of microcytic anaemia, elevated or normal platelet counts, hypolobulation of megakaryocytes, and a low risk of leukaemic transformation, it identifies the 5q- syndrome (Van den Berghe et al., 1974). This occurs mainly in elderly women.

Monosomy 7/7q-

Monosomy 7 or 7q- accounts for 19% of all abnormal karyotypes in primary myelodysplastic syndrome (Mufti, 1992). Monosomy 7 has been associated with Down's syndrome (Bunin et al., 1991), Fanconi anaemia (Stivrins et al., 1984) and familial monosomy 7 (Paul et al., 1987; Gilchrist et al., 1990). The presence of monosomy 7 has been associated with poor prognosis (Nowell, 1992).

Trisomy 8

Trisomy 8 is the most frequent trisomy in human neoplasia and has been shown to account for 194/349 of all cases with a single chromosomal gain as the only change (Heim, Mitelman, 1986). Trisomy 8 is also the most frequent numerical change in MDS and is found in 19% of all cases (Mufti, 1992). The presence of trisomy 8 has been associated with poor prognosis (Yunis et al., 1988; Nowell, 1992).

1.1.1.3.3 Prognostic significance

Patients with MDS and an abnormal karyotype have a shorter median survival and a higher rate of leukaemic transformation than patients with a normal karyotype (Kerkhofs et al., 1987; Pierre et al., 1989; Geddes et al., 1990; Suciu et al., 1990). The presence of complex karyotypic abnormalities is generally associated with a shorter survival rate and a higher leukaemic transformation rate (Pierre et al., 1989; Suciu et al., 1990).

1.1.2 Fluorescence *in situ* hybridization

1.1.2.1 Advances in *in situ* technology

The technique of *in situ* hybridization (ISH) was first described in 1969 (Gall, Pardue, 1969; Pardue, Gall, 1969; Buongiorno-Nardelli, Amaldi, 1970). It allows the detection of specific nucleic acid sequences in biological specimens. It is based on the base pairing of a DNA or RNA probe to complementary sequences in cells of the tissue under investigation. DNA or RNA sequences are first labeled with reporter molecules. The probe and the target DNA are denatured. Complementary sequences are then allowed to reanneal. The probes are then detected either directly or indirectly following hybridization. The earliest methods used radioactively-labeled probes which were then detected by autoradiography. These methods, however. suffered from poor resolution and high background. To overcome these limitations Rudkin and Sollar described in 1977 the modification of ISH using fluorescent detection of chemically modified DNA named fluorescence in situ hybridization (FISH) (Rudkin, Stollar, 1977). In the early 1980s a method for direct labeling of fluorochromes to DNA probes was developed.

1.1.2.2 Probes

A variety of cloned probes are now available for fluorescence *in situ* hybridization. Chromosome-specific repetitive sequence probes, whole chromosome specific probes and unique-sequence probes are now used for metaphase and interphase FISH.

Chromosome-specific repetitive probes

Repetitive elements of DNA have been cloned for all human chromosomes. Repetitive sequences form satellite DNA which occur at or near the centromeres of human chromosomes. When hybridized, chromosome-specific, repetitive probes, produce compact hybridization signals near the centromere or in heterochromatic regions of specific chromosomes. Using these probes it is possible to detect the presence and number of human chromosomes on metaphase spreads and in interphase nuclei.

Whole chromosome probes.

DNA sequences derived from a single human chromosome can be used to detect that chromosome in metaphase spreads and interphase nuclei. Such probes are derived either from somatic cell hybrids containing a single human chromosome or from suspensions of chromosomes purified by flow sorting. When hybridized, whole chromosome probes produce signals that cover nearly the entire length of the chromosome.

Unique-sequence probes

Depending on their size, unique-sequence probes have been cloned using different vectors. Thus plasmid vectors are used for cloning inserts of 1-10 kb, cosmid vectors contain inserts of 35-45 kb and yeast artificial chromosomes (YACs) contain inserts of 200 to 1000 kb. The efficiency of site detection decreases with decreasing probe size.

1.1.3 The advantages and limitations of cytogenetics and FISH

1.1.3.1 Detection of a clone by cytogenetic analysis

The detection of clonal chromosomal abnormalities in patients with haematological disorders enables the distinction to be made between diseases of neoplastic and those of non-neoplastic origin. It constitutes, therefore, a useful diagnostic tool and has

important therapeutic implications. Cytogenetics is the only technique which is routinely used for the detection of clonality in patients with haematological disorders. The criteria used to define 'chromosomally abnormal' or 'cytogenetically clonal' neoplasm were established by ISCN 1985 and they are met when at least three cells with the same chromosomal loss or two sharing the same chromosomal gain or structural abnormality are detected (ISCN(1985), 1985). Applying these criteria cytogenetic analysis is now capable of detecting a chromosomally abnormal clonal population at diagnosis in up to 90% of cases of acute lymphoblastic leukaemia (ALL) (Pui et al., 1990; Secker-Walker, 1990; Dastugue et al., 1992; Van der Plas et al., 1992b; GFCH, 1993), 35%-75% of cases of myelodysplastic syndrome (MDS) (Knapp et al., 1985; Jacobs et al., 1986; Geddes et al., 1990) and 69%-84% of cases of acute myeloid leukaemia (AML) (Misawa et al., 1986; Schiffer et al., 1989; Rowley, 1990). Cases in which a clone is not detected in the bone marrow are either described as chromosomally normal, or as 'fails' depending on the number of metaphases available for analysis. If less than 20 cells are available the case is described as failed.

1.1.3.2 Clonality and chromosomal classification of 'Normal'

It is unclear whether chromosomally normal cases truly lack an abnormal clone, or whether such cases result from the limitations of the cytogenetic technique. Cytogenetic analysis is limited by time constraints to no more than 20 cells. This puts serious limits on the sensitivity of the technique. It has been shown that analysis of 20 metaphases will exclude the presence of an actively dividing ≥11% clone with 90% confidence (Hook, 1977). It follows that clones involving less than 11% of the metaphase population are likely to be missed on routine cytogenetic examination and such cases will be classified as chromosomally normal. This is of particular importance in cases where malignant cells are dividing less efficiently in culture than their normal counterparts. As a result a malignant clone which is underrepresented within the dividing population may escape cytogenetic detection. The existence of malignant clones with low survival properties has been shown in one report. Manabe at al. described a serum -free assay to compare survival requirements of leukaemic B-cell progenitors. In this assay, two cases not giving rise to viable cells in culture were patients with high hyperdiploid clones (Manabe et al., 1992).

1.1.3.3 Clonality and random abnormal cells

Chromosomally normal cases are of two kinds: those in which no abnormal cell is detected and those with random changes i.e. having a single cell showing structural change or chromosomal gain, or no more than two cells with the same chromosomal loss. The presence of a single abnormal cell among 20 cells analyzed raises doubts about its clonal nature. It has been reported that a single abnormal may precede the emergence of a malignant clone. Secker-Walker et al. described a patient in remission from ALL in whom a single cell with monosomy 7 was found in a bone marrow sample. Six months later a clone with monosomy 7 was detected on cytogenetic analysis. This was followed by the emergence of AML (Secker-Walker, Sandler, 1978). McConnel et al described a patient with AML in whom a random trisomy 8 cell was seen several months before full emergence of a clone with trisomy 8 (McConnell et al., 1991).

1.1.3.4 Clonality of cryptic chromosomal abnormalities.

Another aspect of cytogenetic examination which may lead to a diagnostic misinterpretation is the size of the chromosomal lesion. With improved banding techniques it is now possible to detect chromosomal defects as small as 5 Mb. Even though, the gap between cytogenetic sensitivity and the scale of a molecular event remains enormous. The likelihood that there are cases where a chromosomal change is not large enough to produce a visible distortion in a chromosomal banding pattern remains high.

1.1.3.5 Clonality and a chromosomal classification of 'fail'.

Cases in which a clone is not detected and the number of available metaphases is lower than required (usually 20) are classified as 'failed'. The reported failure rate in ALL is as high as 23% to 33% (Jackson et al., 1990; Secker-Walker, 1990; Harris et al., 1992). This leaves a considerable margin of cases in which the cytogenetic diagnosis is not possible due to a lack of dividing cells. It has been shown in one report that the chance to obtain an adequate number of metaphases depends greatly on the quality of sample. Hawkins et al. showed that the likelihood of obtaining 10 or more cells for analysis in adult ALL was as low as 10% for suboptimal samples (Hawkins, Secker-Walker, 1991).

1.1.3.6 Clonality and FISH

Fluorescence *in situ* hybridization (FISH) is a technique which can be used to detect the presence of chromosome abnormalities to which probes are available in both mitotically active and resting cells. In contrast to cytogenetics it is possible to include large numbers of cells in the analysis, making for more sensitive clone detection. In addition the use of unique sequence DNA probes makes it possible to delineate cytogenetically invisible genetic changes. Interphase FISH with chromosome-specific centromeric probes has been used to look for trisomy and monosomy and to determine the presence of clonal chromosome abnormalities in various haematological malignancies. Anastasi et al (Anastasi et al., 1992) used a chromosome 12 specific centromeric probe in cases with chronic lymphocytic leukaemia. FISH confirmed the presence of trisomy 12 in the diagnostic bone marrow samples in 7/7 patients in whom the above abnormality had been previously found by cytogenetics. In addition trisomy 12 was detected in 5 cases: in one case thought to have a normal karyototype, in two cases that had been inadequate for routine cytogenetic analysis, and in two cases that had been found to have an abnormal karyotype without trisomy 12. FISH using chromosome 7 and chromosome 8 specific probes has been applied to detect monosomy 7 and trisomy 8 in patients with myeloid neoplasia (Kibbelaar et al., 1993). In that report most samples with nonclonal metaphase aberrations, including those with only a few metaphases, had increased numbers of aberrant interphase cells: 20% to 80% for -7 and 3% to 43% for +8. In report by Jenkins et al (Jenkins et al., 1992), a chromosome 8 specific probe has been used to detect trisomy 8 in patients with myeloproliferative disorders (MPD), myelodysplastic syndromes (MDS) and acute myeloblastic leukaemia (AML). In 4/13 patients in whom the initial diagnostic cytogenetic analysis revealed a single abnormal metaphase, FISH analysis found significant levels of cells with trisomy 8. Brizard et al (Brizard et al., 1994) reported that FISH using chromosome-specific centromeric probes revealed monosomy 7 which was undetectable by karyotypic study in 5%-22% cells from 15 patients with MDS. In a report by Chen et al (Chen et al., 1993b), in 10/10 patients with a single trisomic cell on metaphase analysis (random trisomies) FISH detected the presence of a clone in interphase cells indicating that these 'random' abnormalities were in fact clonal. The use of unique-sequence probes to detect clonality has been demonstrated in a report by Van Rhee et al (Van Rhee, Kasprzyk, 1995). A patient with ALL was found to have

only chromosomally normal metaphases on a routine cytogenetic examination. However, when probes containing fragments of *BCR* and *ABL* genes were used the juxtaposition of the two genes on the apparently normal chromosome 22 was demonstrated.

Thus, FISH can be successfully used for the detection of numerical as well as structural chromosome abnormalities. The main disadvantage of FISH is the fact that it is incapable of a comprehensive description of all chromosomal abnormalities occurring in the malignant clone. It is instead guided by cytogenetic analysis.

In summary, conventional cytogenetics of haematological malignancy has proved to be a valuable tool in haematological practice. It has however important limitations. FISH which is at present time incapable of replacing cytogenetics in its diagnostic task has several advantages. The sensitivity of FISH and the fact that it does not need dividing cells makes it an ideal candidate to investigate cases where cytogenetics was ambiguous or unavailable.

1.2 CLONALITY AND LINEAGE INVOLVEMENT IN HAEMATOLOGICAL MALIGNANCY

1.2.1 Normal haemopoiesis

1.2.1.1 Stem Cell Model

The modern concept of haematopoiesis is based on the stem cell model which assumes that all blood cells are derived from a pool of primitive progenitor cells called pluripotent stem cells. Pluripotent stem cells are capable of proliferation, differentiation, self-renewal and give rise to a series of committed progenitor cells for all blood cell lineages. Committed progenitor cells, which are capable of extensive proliferation without self-renewal, give rise after a number of cell divisions and differentiation steps to mature cells (McCulloch, 1983).

The existence of myeloid and lymphoid progenitors has been demonstrated in mice (Curry et al., 1967; Abramson et al., 1977) and in humans (Messner et al., 1981) by *in vitro* culture techniques. Stem cells have also been demonstrated using glucose-6-phosphatase (G6PD) isoenzymes analysis in humans (Fialkow et al., 1977; Martin et al., 1980).

Early myeloid committed progenitor cells are identifiable by *in vitro* assays (Johnson, Metcalf, 1977; Fauser, Messner, 1979; Messner et al., 1981; Gordon, 1993). These comprise mixed myeloid progenitor cells capable of giving rise to all myeloid lineages (CFU-GEMM), granulocyte-monocyte progenitors (CFU-GM), erythrocyte progenitors (CFU-E), megakaryocyte progenitors (CFU-Mk), basophil progenitors (CFU-B) and eosinophil progenitors (CFU-Eo). Late myeloid committed progenitor cells are identifiable by staining techniques and consist of myeloblasts, megakaryoblasts and erythroblasts. Mature myeloid cells are also identifiable by staining techniques both in bone marrow and peripheral blood and consist of basophils, eosinophils, neutrophils, erythrocyte, megakaryocytes and monocytes.

The evidence for subsequent stages in lymphopoiesis, which result in the formation of B and T lymphocytes, has come from immunophenotype and molecular analysis of lymphoid malignancies. The concept of lymphopoiesis is based on the hypothesis that the leukaemic cells are the neoplastic counterparts of normal lymphoid progenitors 'frozen' at various stages of their development.

The *in vitro* conditions to for the myeloid precursor (CFU-GEMM) are well established. In contrast, the existence of its lymphoid counterpart capable of giving rise to both B and T lymphocytes has not been proven as yet. There is however indirect evidence that such a lymphoid precursor may really exist. The evidence came from immunoglobulin gene (*IGH*) rearrangement studies. These have shown the presence of rearranged *IGH* genes in both T and B lymphocytes in mice (Kemp et al., 1980; Kurosawa et al., 1981) and in human lymphoid tumors (Pelicci et al., 1985)

The subsequent stages of B cell development consist of the following stages: a proB, pre-B and mature B-cell. The pro-B stage is characterized by the DR and CD34 expression. Both, heavy and light chain immunoglobulin genes are in the germline configuration. The pre-B stage is characterized by the expression of CD10, CD19 and CD34 which is later on replaced by CD22, CD20 and surface IgM. Heavy-chain immunoglobulin genes are rearranged at this stage. During the B-cell stage both heavy and light immunoglobulin genes are rearranged and the cell expresses CD19, CD20, CD21, CD22. The mature B-cell stage is characterized by the expression of surface immunoglobulins (first IgM, then IgM plus IgD, then other isotypes) (Foon, Todd, 1986; Pui et al., 1993).

The sequence of events in T-cell development, originally proposed by Reinharz (Reinherz et al., 1980) and based on immunological data was later modified and enriched with data from T-cell receptor gene rearrangement studies. T-cell ontogeny divide into four stages: stage I-'double negatives' (CD4 -ve and CD8 -ve) with expression of CD3, CD2, CD7, TdT and beta chain T-cell receptor genes in germline position; stage II-'double positives' with CD4 and CD8 expression and beta chain genes rearranged; stage III with the retention of CD4 and CD8 but without TdT and stage IV with the expression of either CD4 or CD8 and fully assembled alpha/beta T-cell receptor complexes (Foon, Todd, 1986; Pui et al., 1993).

1.2.1.2 Control of haematopoiesis

Haematopoiesis maintains the number of cells of all lineages at a constant level during steady state but it is also capable of responding to an environmental stimulus (bacterial infection, hypoxia) by elevating the production of cells of a specific lineage. It follows that tight control over cell production (proliferation), specialization (differentiation, commitment) and programmed cell death (apoptosis) is necessary. The important role

in the control of haematopoiesis is played by haematopoietic growth factors (HGF) and haematopoietic microenviroment (HM)

Haematopoietic growth factors

Haematopoietic growth factors (HGFs) are the glycoprotein hormones that regulate the proliferation and differentiation of committed progenitors and the function of mature blood cells. The HGFs identified in *in vitro* colony assays are called colony stimulating factors (CFSs) and those derived from lymphocytes are termed interleukins (ILs). The main functions of HGFs are stimulation of cell survival (prevention of apoptosis), proliferation and differentiation. More mature cells may also respond to HGFs by increased functional activity and or production of other growth factors (Metcalf, 1989; Nicola, 1989; Cross, Dexter, 1991). Dependent on the cell stimulated, the growth factors are classified as multilineage and lineage restricted HGFs. Multilineage HGFs comprise stem cell factor (SCF), granulocyte, monocyte colony stimulating factor (GM-CSF) and interleukin 3 (IL-3). Lineage restricted HGFs comprise IL-5 (acting on eosinophilic lineage), IL-6 (restricted to megakaryocytic haemopoiesis), granulocyte colony stimulating factor (G-CFS) (stimulating granulopoiesis), erythropoietin (Eo) (stimulating erythropoiesis) and monocyte CSF (acting on monocyte precursors).

All HGFs act via a common signalling pathway that consists of cell surface receptors, and intercellular signalling devices. Two types of HGF's receptor have been described. The first type (M-GCF receptor) has an extracellular, ligand-binding domain and an intracellular, tyrosine kinase domain responsible for transducing the signal (Ullrich, Schlessinger, 1990). The second type of receptor, common for Il-3, GM-CSF, IL-5 contains several subunits but does not contain protein tyrosine kinase (PTK) (Miyajima et al., 1993).

Haematopoietic microenviroment

The haematopoietic microenvironment is the place of haematopoietic cell development. The haematopoietic microenvironment consists of macrophages, endothelial cells, fibroblasts and adipocytes (Du, Williams, 1994). The potential functions of the haematopoietic microenvironment are: the stabilization of growth factors by binding to extracellular matrix molecules or membrane proteins, the production of both positive and negative regulators of haematopoiesis and colocalizations of haematopoietic cells and growth factors allowing receptor modulation by small quantities of cytokines (Williams, 1993).

1.2. 2. Malignant haematopoiesis

1.2.2.1 Stem cell model and malignant haematopoiesis

Although the outcome of neoplastic haematopoiesis is different from its normal counterpart the stem cell model is still applicable for the description of this kind of haematopoiesis. Experiments with clonogenic AML cells *in vitro* have revealed a small proportion of actively dividing cells that correspond to the haematopoietic cell progenitors (Griffin, Lowenberg, 1986; Lowenberg, Touw, 1993). Experiments with B-precursor leukaemia have also shown that subpopulations of cells at an early stage of differentiation show a clonogenic advantage, which is typical of progenitor cells (Estrov et al., 1993; Ashley et al., 1994; White et al., 1995). Also, experiments with C-ALL, revealed subpopulations with a less mature phenotype (Ryan et al., 1986) and showed that these had greater clonogenic activity (Ashley et al., 1994).

1.2.2.2 Clonality

In contrast to normal haemopoiesis which is multiclonal and contain members of 10 to 25 clones as demonstrated by Fialkow (Fialkow, 1973), leukaemopoiesis is monoclonal. Haematological malignancy results from proliferation of the progeny of a single cell. The first indication of that was the observation in multiple myeloma that the secreted immunoglobulin was electrophoretically homogenous. The concept of monoclonality has finally become established after the work of Fialkow using specific markers of clonality such as glucose-6-phosphatase (G6PD). By analysis of G6PD isoenzymes a clonal origin has been demonstrated for the tumour cells in several haematological diseases.

Techniques used for the detection of clonality

In addition to G6PD isoenzymes analysis, evidence for the monoclonal origin of neoplasia has also come from the application of many other techniques. Techniques which have demonstrated the monoclonal nature of neoplasia include: X-linked restriction fragment length polymorphism (RFLP), light chain restriction, immunoglobulin and T-cell receptor rearrangements, cytogenetics, a combined morphology, antibody, chromosome (MAC) technique and fluorescence *in situ* hybridization (FISH).

Glucose-6-phosphatase (G6PD) isoenzymes analysis

This technique has been widely used in order to establish the monoclonal origin of many haematological malignancies. The G6PD enzyme gene is located on the X chromosome. The enzyme occur in two forms A and B. Heterozygous females have both enzymes. According to the Lyon hypothesis, one of the two X chromosomes becomes inactivated. Some somatic cells will express G6PD A and some G6PD B. The G6PD isoenzymes analysis of a given tissue is accomplished by determining the ratio of two G6PD isoenzymes A and B. Polyclonal tissue will show both isoenzymes, while monoclonal only one either A or B.

X-linked restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) indicates the variation in the size of a DNA fragment, detectable with a given probe, after digestion with a restriction enzyme. The combination of various RFLPs along one chromosome is called the haplotype. This technique is used to establish monoclonal or polyclonal origin of a given tissue in women having different RFLP haplotypes ('informative' women). Similar to the G6PD analysis, X-linked RFLP analysis of monoclonality is based on the Lyon hypothesis. Polyclonal tissue will show two different RFLP haplotypes, monoclonal only one haplotype.

Immunoglobulin and T-cell receptor rearrangements

This technique is applicable to lymphoid tissue only. The mono or polyclonal origin of tissue can be determined by examining immunoglobulin (*IG*) or T cell receptor (*TCR*) gene rearrangement patterns. In a polyclonal tissue a diversity of rearrangements will be present while monoclonal populations will have only one rearrangement.

Light chain restriction

This technique is applicable to B-lymphoid tissue only. A mature B lymphocyte expresses a surface immunoglobulin which contains either κ or λ light chain In a polyclonal population of B lymphocytes both κ and λ immunoglobulin light chain will be detected, in a monoclonal only one, either κ or λ.

Cytogenetics

This technique is now routinely used for the determination of clonality in haematological malignancy. The clonal status of a given tissue is established when two cells with the same structural change or chromosomal gain, or three cells sharing the same chromosomal loss are found.

Morphology, Antibody, Chromosome (MAC)

Morphology, Antibody, Chromosome (MAC) technique can be used to identify chromosomal abnormalities in cells which had been previously morphologically or immunologically stained (Teerenhovi et al., 1984; Teerenhovi et al., 1986; Knuutila, Teerenhovi, 1989). The method differs from conventional cytogenetics in that after hypotonic treatment, the cells are cytocentrifu ged onto glass slides. In mitotic cells, this procedure results in an adequate spread of the chromosomes within the intact cell membrane. The cytoplasmic structure also remains intact, so that cytogenetic preparations are of good quality. Morphologic and immunologic identification of mitotic cells can be done using routine haematological stains, such as Giemsa or Sudan black B, and various antisera using immunofluorescence or alkaline phosphatase anti-alkaline phospahatase (APAAP) technique. The chromosomes can be simultaneously analyzed on slides stained with Q or G-banding. Such an approach enables the detection of chromosomal abnormalities in particular cell lineages.

Fluorescence *in situ* hybridization (FISH)

Certain chromosome abnormalities can be detected in interphase cells using DNA probes with fluorescent *in situ* hybridization (FISH). When interphase FISH is combined with morphology or immunophenotyping it is possible to establish in which cell lineages the chromosomal abnormalities occur. FISH can be carried out on peripheral blood or bone marrow smears which have been previously stained and morphologically classified. Selected cells must be photographed and then relocated after hybridization (van Lom et al., 1993; Nguyen et al., 1994). For the simultaneous detection of immunophenotype and chromosome abnormalities the APAAP technique for immunophenotyping has the advantage that it is visible with light as well as with fluorescent microscopy (Price et al., 1992).

1.2.2.3 Leukaemic progenitor cell

G6PD isoenzymes analysis, X-linked RFLP, MAC and FISH have all been used to determine the extent of clonality in different types of haematological malignancy. The detection of clonality in a particular cell lineage provides circumstantial evidence for the type of haematopoietic progenitor cell in which the malignant transformation has taken place. Thus, the greater the number of cell lineages involved the more pluripotent is the leukaemic progenitor cell.

The origin of myeloproliferative disorders

All myeloproliferative disorders (MPDs) are thought to arise in a pluripotent stem cell. The presence of a single G6PD isoenzyme has been detected in the blood cells of women with CML who are heterozygotes for isoenzymes A and B (Fialkow et al., 1967; Fialkow et al., 1977; Koeffler et al., 1980). The cells include red cells, neutrophils, eosinophils, basophils, monocytes and platelets. Fibroblasts and other somatic cells showed the presence of both isoenzymes. In some cases the involvement of B lymphocytes has also been shown (Martin et al., 1982). When the analysis of G6PD isoenzymes was applied to patients with polycythemia vera (PV) a single isoenzyme type was detected in granulocytes and platelets (Adamson et al., 1976). Using similar methods Raskind et al have also demonstrated the involvement of B-cells (Raskind et al., 1985).

The origin of myelodysplastic syndrome

The origin of MDS is still debatable. A variety of techniques such as chromosome analysis, X-linked RFLP and PCR amplification of point mutations in the ras gene have shown that MDS is a clonal disease which has arisen in a multipotent stem cell. The abnormal clone has been shown to give rise to granulocytes, monocytes and B and T- lymphoid cells (Janssen et al., 1989). In contrast, FISH of interphase peripheral blood cells with probes for chromosomes 7 or 8 in patients with monosomy 7 or trisomy 8 respectively has shown that chromosome abnormalities were not present in lymphoid cells (Gerritsen et al., 1992; Anastasi et al., 1993).

The origin of acute myeloid leukaemia

Studies of female patients with AML who are heterozygous at the G6PD locus indicate that virtually all cases of AML at clinical presentation are monoclonal (Fialkow et al., 1979; Fialkow et al., 1981). It is now assumed that AML arises in a committed myeloid progenitor cell. The exact level of transformation, however, is disputable. A G6PD analysis of cells derived from different haematopoietic lineages in individual AML patients indicated that, in some cases, the disease was restricted to granulocyte/monocyte progenitors. Other cases suggested a multipotent myeloid progenitor giving rise to cells of the granulocyte, erythroid and megakaryocytic lineages (Fialkow et al., 1979; Fialkow et al., 1981). These studies reflect heterogeneity in the cell of origin in different cases of AML. In some patients the disease appears to be restricted to cells of the granulocyte/monocyte lineages which would indicate that

transformation has occurred at CFU-GM level. In other cases the disease involves, in addition, erythroid and megakaryocytic lineages suggesting that the transformation has occurred at CFU-GEMM level. None of the studies, however, indicated the involvement of the lymphoid lineage which would have suggested that the target cell was a multipotent stem cell. Myeloid restriction of AML was also reported in the studies where certain chromosomal abnormalities were used as markers of clonality (Keinanen et al., 1988; Stamberg et al., 1988; Baurmann et al., 1993; van Lom et al., 1993; Kwong, Chan, 1994).

The origin of acute lymphoblastic leukaemia

ALL is regarded as the result of a somatic mutation in a committed lymphoid progenitor cell. Dow et al (Dow et al., 1985) investigated isotypes of the glucose-6-phosphate dehydrogenase (G6PD) isoenzymes in 19 females heterozygous for this X chromosome-linked enzyme. The lymphoid blast cells from all cases displayed a single, (either A or B G6PD) isoenzyme type while in other cell lineages both A and B G6PD types were observed. The lack of myeloid involvement was also confirmed in the studies where certain chromosomal abnormalities were used as markers (Knuutila et al., 1993; Martin-Henao et al., 1994).

The origin of the acute leukaemias may be heterogeneous.

The assumption that acute leukaemia arises in a cell committed to lymphoid (ALL) or myeloid (AML) development cannot be however regarded as firmly established. It has been shown that in a proportion of Ph+ ALL the abnormality was detected not only in lymphoid but also in cells of myeloid origin (Tachibana et al., 1987; Kalousek et al., 1988; Dow et al., 1989; Raimondi et al., 1990; Jackson et al., 1992). Similarly, the existence of so called 'hybrid' or 'mixed' leukaemias, showing features of both myeloid and lymphoid lineages, seems to contradict the above presumptions. Mixed lineage (biphenotypic) leukaemia refers to cases in which both lymphoid and myeloid-associated surface antigens are expressed on the same cells. The incidence of mixed lineage leukaemia has been estimated to be as high 15% to 20% in some studies but generally ranges from 1% to 10% of ALL (Mirro et al., 1985; Hoelzer, Gale, 1987) and 16.8% of AML cases (Pui et al., 1991). Mixed leukaemia has been strongly associated with the 11q23 abnormalities and the Philadelphia translocation (Drexler et al., 1991; Hudson et al., 1991; Cuneo et al., 1992; Cuneo et al., 1993). There are two hypotheses aiming to explain the origin of 'mixed' leukaemias. The basic concept of the so called

'lineage infidelity' theory is that the leukaemic cell has an aberrant combination of markers as a consequence of its leukaemic developmental programme. This model was originally suggested by McCulloch following the demonstration of erythroid and myeloid markers in several leukaemic cell lines (Marie et al., 1981). These observations were later extended to AML blasts that had cytoplasmic immunoglobulin and surface myeloid antigen (Smith et al., 1983). An alternative explanation was proposed by Greaves (Greaves et al., 1986). According to his 'lineage promiscuity' theory, commitment to different lineages may be an inefficient process and certain cells may retain features which are normally lost during commitment to a lineage. Alternatively certain cells may actually develop features of a separate lineage from that to which they are committed. It is suggested that the leukaemic process will 'immortalise' an otherwise non-viable marrow precursor in a pre-commitment phase of haematopoiesis. Coexpression of different lineage markers may, as a consequence, be associated with leukaemic blasts.

1.2.3 Leukaemic stem cell and chemocurability

Modern treatment regiments have produced striking differences in therapeutic success between different leukaemias. Thus, 70% of children with ALL are now expected to achieve long remission and probably cure (Chessells, 1992). In contrast, long term remission in adults with ALL is expected in no more than 30% of patients (Secker-Walker et al., 1997). Similar figures (16%-28%) are quoted for patients with AML (Ben-Bassat et al., 1993). The reason why these differences occur is a subject of a much speculation. Pinkel suggests that it is the genotype of the leukaemia that determines its response to therapy, but not the cell type (Pinkel, 1987). He argues that the genetic alterations underlying leukaemic cell transformation disrupt the characteristics of the cell to an extent that a clear relationship to a normal cell cannot be ascertained. He argues that chemotherapy of a given leukaemia should be dictated by the chromosomal or/and genetic make-up, rather by the presumed cell type. To support his hypothesis he quotes the well known fact that leukaemia can be stratified into different prognostic groups according to chromosomal abnormalities e.g. hyperdiploidy or Ph+ ALL. A different view is held by two authors. Jasmin at al (Jasmin, 1988) suggests that the low success rate in CML, MDS and adult acute leukaemia is the result of the stem cell origin of the tumour. He argues that haematological malignancies of

stem cell origin are chemo-incurable and can only be cured by bone marrow transplantation. A proportion of the acute leukaemias, occurring predominantly in children and which are chemo-curable, are derived from the compartment of haemopoietic progenitors already irreversibly committed to a single lineage. A similar view is held by Greaves. In his report Greaves hypothesises that most childhood acute lymphoblastic leukaemias and some other paediatric cancers are chemo-curable because they arise in cell populations which are functionally transient, chemosensitive and programmed for apoptosis. Greaves argues that most adult acute leukaemias are chemo-incurable, at least in part because they originate in relatively drug resistant pluripotent stem cells with extensive self renewal capacity. The latter property, in turn, increases the probability of clones evolving with multi-drug resistance (Greaves, 1993).

However, the association between stem cell leukaemia and prognosis seems to be more complicated. Secker-Walker et al. reviewed the prognostic implications of the lineage heterogeneity in Ph+ ALL (Secker-Walker, Craig, 1993). Patients with myeloid as well as lymphoid involvement of clonal cells had significantly longer survival than patients with lymphoid involvement only. This was later confirmed by Anastasi et al. (Anastasi et al., 1996). Paradoxically, this finding is contrary to the theoretically predicted features of stem cell leukaemia (Jasmin, 1988; Greaves, 1993). Further studies on the issue are required. Meanwhile, the therapeutic implications of the level of commitment of the leukaemic target cell has emerged as an important question.

1.3 THE DETECTION OF MINIMAL RESIDUAL DISEASE IN ACUTE LYMPHOBLASTIC LEUKAEMIA

The current treatment of patients with ALL results in a high remission rate, especially in childhood. However, many of those who achieve complete remission will eventually relapse. It has been shown that most relapses originate in neoplastic cells that are a part of the clone seen at diagnosis (Borella et al., 1979; Dow et al., 1985; Pui et al., 1986; Raghavachar et al., 1987; Steward et al., 1994). It follows that leukaemopoiesis continues during remission.

Complete remission (CR) is clinically defined as not more than 5% of blasts in a patient's bone marrow. The leukaemic cells which persist in the bone marrow of a patient in remission are termed minimal residual disease (MRD). It has been shown that the presence of 5% blasts in the bone marrow may represent a leukaemic burden of MRD as high as 10^{10} blasts (Van Bekkum, 1984).

The importance of MRD detection in patients in complete remission lies in the possibility that it may be possible to establish a relationship between the size of the clone and risk of relapse. The ability to detect and quantitate MRD in ALL and to relate this to the risk of relapse would provide the means of monitoring the effectiveness of treatment and adjusting therapy when necessary. Although the exact link between the number of leukaemic cells and patient's prognosis has not yet been established, some evidence exists that the detection of low levels of leukaemic cells may precede the full re-emergence of the clone and subsequent relapse (Campana et al., 1990; Anastasi et al., 1991a; Potter, 1992; Cole-Sinclair et al., 1993; Nizet et al., 1993; Cave et al., 1994; Nylund et al., 1994; Kitchingman, 1995).

1.3.1 Methods for MRD detection

Leukaemic cells can be distinguished from normal haematopoietic progenitors on the basis of morphologic and cytochemical properties, karyotypic or genetic abnormalities, antigen-receptor gene rearrangements, cell growth requirements in vitro and immunophenotype. Combinations of these characteristics have been exploited to detect small numbers of neoplastic blasts among normal cells.

1.3.1.1 Morphology

The morphological approach for the detection of MRD involves microscopical evaluation of the bone marrow or extra-medullar sites such as the central nervous system and testis. In the bone marrow, the presence of normal blasts limits the sensitivity of this approach to 5%. A common problem which is encountered with morphological scanning for MRD in childhood ALL, is caused by the presence of a rebound lymphocytosis at the end of chemotherapy. This may lead to a false conclusion about impending relapse (Borella et al., 1972). Attempts to monitor disease by serial examinations of the bone marrow throughout chemotherapy have been disappointing (Komp et al., 1983; Rogers et al., 1984). One such study yielded 75% of false negative results (Komp et al., 1983). Problems have also been encountered when examining extramedullary sites. In the examination of cerebro-spinal fluid, false positives may occur due to contamination with peripheral blood, or cell morphology may be altered by centrifugation, viral infection or chemotherapy (Odom et al., 1990). The detection of MRD in testis has been complicated by high false negative rates resulting from sampling errors and the difficulty in distinguishing between testicular stromal cells, lymphocytes and lymphoblasts (Hudson et al., 1985; Pui et al., 1985).

1.3.1.3 Cytogenetics

Cytogenetics plays an important role in the diagnosis and prognosis of ALL. Improved cytogenetic methods allow detection of a chromosomally abnormal clonal population at diagnosis in up to 90% of cases (Pui et al., 1990; Secker-Walker, 1990; Dastugue et al., 1992; Van der Plas et al., 1992b; GFCH, 1993). However, the tracking of MRD with this technique suffers from serious limitations. The cytogenetic approach is restricted by time constraints to analysis of no more than 20 cells. This puts a serious limit on the sensitivity of the technique. It has been shown that analysis of 20 metaphases will exclude the presence of an $\geq$11% clone with 90% confidence (Hook, 1977). This is much higher than the 5% limit of the definition of clinical remission and only clones with a high mitotic rate are likely to be detected.

The main advantage of the cytogenetic approach is its flexibility. Cytogenetics unlike many other methods does not have to rely solely on the abnormalities detected at diagnosis. Providing clonal evolution has not involved loss of all the chromosomal

abnormalities, leukaemic cells can be still identified, even though their chromosomal 'make up' may have changed.

1.3.1.4 Immunology

Immunological methods represent a rapid option for investigation of MRD in a significant proportion of patients with ALL. This technique relies upon detection of leukaemia-associated immunophenotypes which were detected at diagnosis on leukaemic blasts. These can be then used to detect MRD at various levels of sensitivity. The potential sensitivity of detection of residual disease expressing any combination of antigens is determined by the background level of normal cells that express the same markers. Single antigens are not suitable for distinguishing neoplastic from normal lymphohaematopoietic cells, because the same antigens found on malignant cells are also present on their normal counterparts (Janossy et al., 1979; Greaves et al., 1980). To overcome this problem double and triple staining techniques have been developed. The detection of MRD in T-ALL using double staining has proved to be particularly effective. The combination of antigens which are found on T-ALL blasts, CD3/TdT, occur in 90% of T-ALL and are very rare in normal marrows (Campana, Pui, 1995). These combinations of antigens allow the detection of MRD with a sensitivity of $\leq$ 0.01% (Campana et al., 1990). In B-lineage ALL, TdT is expressed in association with Cμ, CD21, CD33, Cdw65, CD13 or CD2 (Campana et al., 1990; Campana et al., 1991). The detection of MRD in B-lineage ALL using double staining is not as sensitive as its T-lineage counterpart. Thus, CD13/TdT or CD33/TdT positive cells constitute 5%-10% of the normal bone marrow population, while marker combinations of cμ/TdT or CDw65/TdT occur in up to 20% of the TdT population (Campana et al., 1990). The combination of TdT/ Cμ, which occurs most frequently in childhood ALL is present on cells found in the marrows of healthy infants and on marrows which are regenerating, and the sensitivity of detection using this marker combination is only 10^{-3} (Campana et al., 1990; Campana et al., 1991). The use of triple staining techniques can markedly improve the sensitivity of MRD detection in B-lineage ALL. There are at least 11 triple antigens combinations which are now being used as MRD targets and are sufficiently sensitive to detect between 0.01% and 0.03%$\pm$ 0.02% malignant cells (Campana, Pui, 1995).

1.3.1.5 Polymerse Chain Reaction (PCR)

The introduction of PCR has set new standards for the analysis of MRD in patients with leukaemia by allowing the identification of as few as one malignant cell in a background of 10^5 or 10^6 normal cells (Fey et al., 1991; Sklar, 1991; Malinge et al., 1992; Potter, 1992; Thompson et al., 1992; Cross et al., 1993). There are two marker categories which are used for MRD investigation: (i) disease-specific chimeric genes, created by the fusion of two genes at the breakpoints of chromosomal translocation and (ii) clone-specific junctional regions generated physiologically through immunoglobulin (Ig) or T-cell receptor (*TCR*) gene rearrangements.

Disease-specific gene fusion in chromosomal translocations.

Tumour-specific chromosomal translocations provide ideal disease markers that can be detected by molecular means in subgroups of ALL. At least 11 disease-specific fusion regions have now been cloned and become the targets for MRD investigation using PCR. These include *BCR-ABL* created in t(9;22)(q34;q11), *E2A-PBX1* created by t(1;19)(q23;p13), *MLL-AF4* found in t(4;11)(q21;q23), *IL3-IGH* found in t(5;14), *MLL-AF9* found in t(9;11), *E2A-HLF* found in t(17;19) and *MYC-IgH* found in t(8;14). The molecular targets in T-ALL include *RHOM2-TCR*γ found in t(11;14), *TAL1-TCR*α found in t(1;14) and *HOX11-TCR*α found in t(10;14) (Campana, Pui, 1995).

Immunoglobulin or T-cell receptor rearrangements

In addition to leukaemia specific chromosomal translocations, leukaemia-non-specific clonality markers such as the junctional region of the rearranged *IG* and *TCR* genes can be investigated by PCR. This is based on the fact that the junctional regions vary considerably due to the joining of different variable (V), diversity (D), and joining (J) gene segments as well as the random insertion and deletion of nucleotides at the joining sites. Such rearrangements are clonal. Demonstration of immunoglobulin and T-cell receptor (*TCR*) gene configurations at diagnosis provides a means of tracking the malignant clones throughout the disease. The main advantage of this approach is that it is wide applicable. Nearly all cases of ALL are characterized by a unique, clonal rearrangements of the immunoglobulin heavy chain (*IGH*) or the T-cell receptor (*TCR*) genes. The limitation of this approach is that the target loci may be deleted, and additional rearrangements may become dominant (clonal evolution) during the course of the disease (MacIntyre et al., 1989; Kiyoi et al., 1992; Tycko et al., 1992; Steward et al., 1994).

1.3.1.6 Fluorescence in situ hybridization (FISH)

Fluorescence *in situ* hybridization techniques rely on chromosome-specific and gene-specific DNA probes to identify numerical and structural chromosome abnormalities. The main advantage of this approach is that it enables the detection of chromosomal abnormalities in interphase cells, greatly increasing the sensitivity over that of the chromosomal method. It is however markedly less sensitive than RT-PCR for the detection of aberrant fusion products.

The sensitivity of the interphase FISH assay is limited by false positives. The frequency of false positives varies according to the particular chromosomal abnormality and the probe or set of probes used for their detection. The presence of a clone is only inferred if the number of positive cells in the sample being investigated is greater than the mean + twice the standard deviation of those in the control samples. This sets the limit of the sensitivity of the technique. The sensitivity for the detection of monosomy has been shown to be quite low allowing the detection of the clone which is at least 5% up to 18% of the sample (Kibbelaar et al., 1993; Brizard et al., 1994). The sensitivity for the detection of *BCR-ABL* translocation has been shown to be 0.5% - 10% (Arnoldus et al., 1990; Tkachuk et al., 1990; Chen et al., 1993a; Dewald et al., 1993; Amiel et al., 1995). The sensitivity for the deletion of the retinoblastoma gene has been reported to be 12% (Stilgenbauer et al., 1993). The range of 0.5%-4% has been reported for the detection of the single trisomies (Rivera et al., 1991; Anastasi et al., 1992; Jenkins et al., 1992; Chen et al., 1993b; Escudier et al., 1993; Kibbelaar et al., 1993; Brizard et al., 1994).

FISH has been used with various types of probes to investigate the presence of MRD. Using FISH with an X chromosome-specific probe the detection of 1.6% of interphase cells bearing trisomy X has been reported in the remission bone marrow sample of a patient with ALL (Anastasi et al., 1991a). The same group followed two children with ALL and trisomy 17 (Anastasi et al., 1991b). The investigation of remission bone marrow from these patients with alpha centromeric chromosome 17 specific probe made it possible to distinguish between leukaemic (triploid) and non-leukaemic (diploid) cells. Nylund et al used FISH to investigate MRD in interphase as well as metaphase cells of patients with various haematological malignancies (Nylund et al., 1994). The author detected MRD in 3/7 patients with ALL. The persistence of MRD in ALL with high hyperdiploidy was reported in two studies. Hereema et al. examined

bone marrow samples from eight patients at early clinical remission. One month after diagnosis, significant numbers of cells with chromosome gains were detected in 2/7 cases tested. (Heerema et al., 1993). Two months after diagnosis trisomic and tetrasomic cells were within control values. White et al. conducted 161 analyses of remission bone marrow aspirates in 13 children with hyperdiploid ALL (White et al., 1995). MRD was detected in 5/13 cases of ALL investigated while on chemotherapy. One out of five newly diagnosed cases and all three relapse cases of ALL had significantly raised levels of hyperdiploid cells in the day-28 bone marrow aspirates.

1.4 AIMS OF THE STUDY

The size of the malignant clone in haematological malignancies at diagnosis and the level of commitment of the target cell for malignant change both determine the extent and severity of the disease and may play an important role in the patient's response to treatment. Persistence of the clone in remission indicates the presence of minimal residual disease (MRD) which may require therapeutic intervention. The size of the clone can be judged from chromosome analysis of a bone marrow sample but this estimate may differ widely from the size of the clone in the bone marrow as a whole. Cytogenetic analysis provides information only about the small fraction of cells undergoing cell division. Furthermore cytogenetics gives no information about the types of cell in which the clone is to be found. The principle aim of this thesis was to gain insight into the size and location of the malignant clone in non-dividing cells from patients with a haematological malignancy. The findings are compared with our knowledge of the extent and 'dynamics' of abnormal clones as indicated by cytogenetic studies. The investigations of leukaemic bone marrow comprise three topics.

Investigation of the presence of a clone in chromosomally failed or normal samples and of the clonal status of a single abnormal cell in a population of normal cells.

Cytogenetics, in a proportion of cases, fails (due to the lack of a clone in insufficient dividing cells) or results in the detection of only chromosomally normal cells. In other cases cytogenetic analysis results in the detection of one or more abnormal cells which do not constitute a clone. Such results are of no diagnostic value. The aim of the first study was to test the hypothesis that chromosomally abnormal clones occur in some of these cases, that the clones are present only in the resting cells and that FISH can be successfully used to detect clones in non-dividing cells.

Investigation of the cell types which belong to the clone in acute leukaemia.

The level of commitment of the target cell for chromosomal change can be judged by the different cell lineages belonging to the clone. The aim of this project was to examine the extent of lineage involvement in malignant clones with specific chromosome abnormalities in patients with acute lymphoblastic leukaemia (ALL) or acute myeloid leukaemia (AML) and hence infer whether the target cell was a pluripotent stem cell or a lineage committed progenitor cell.

The detection of minimal residual disease in patients with ALL and a high hyperdiploid clone.

A high hyperdiploid clone (with gain of 4 or more chromosomes) is detected at diagnosis in 30% of children and up to 10% of adults with ALL. For several years a sensitive method of MRD detection (RT-PCR) able to detect 10^{-4} to 10^{-6} cells has been available only for patients with one of the translocations which have been cloned and sequenced. The aim of this study was to develop and apply an equally sensitive FISH method for MRD detection of hyperdiploid clones in patients with ALL.

Chapter 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.2 Patients material

Samples of bone marrow which had been taken for clinical reasons at diagnosis or on follow up were investigated in this study. Two types of such samples were used in the study. (i) Samples remaining from cytogenetic investigation which had been prepared for chromosome analysis at diagnosis or during the course of leukaemia and were surplus to requirements. These archived samples had been stored in fixative at 4 ^{o}C for periods ranging from 1 to 84 months. (ii) Freshly aspirated bone marrows which had been taken for clinical assessment. Bone marrow aspirates were collected into sterile tubes containing heparin as a anticoagulant or full culture medium as follows: RPMI medium, 50 IU/ml penicillin, 50μg streptomycin (GIBCO, UK) and 10 U/ml heparin.

2.1.2 YAC culture media materials

Yeast N_2 base, casein hydrolysate came from DIFCO, ammonium sulfate from BDH, adenine sulfate, sorbitol and sarkosyl from SIGMA.

2.1.3 FISH materials

2.1.3.1 Probes

The probes and their source are listed in table 2.1

2.1.3.3 FISH reagents

Sodium chloride and sodium citrate came from BDH. FITC-avidin, FITC-avidin/rhodamine-labeled anti digoxigeneine, anti-avidin antibody and propidium iodide came from Oncor.

2.1.4 Morphology/immunology materials

2.1.4.1 May-Grunwald-Giemsa (MGG) staining

May-Grunwald-Giemsa came from Sigma

2.1.4.2 Alkaline-phosphatase anti-alkaline-phospatase (APAAP) technique

Antibodies

The following monoclonal antibodies were purchased from DAKO: T-lymphoid (CD3), B-lymphoid (CD19), myeloid (CD13) and erythroid (Glycophorin A). The Rabbit anti-mouse antibodies came also from DAKO.

APAAP reagents

APAAP complex was purchased from DAKO, alkaline-phosphatase substrate from SIGMA.

Table 2.1
Probes used in the study

Name	Reporter molecule	Locus	Source
D4Z1	biotin	chromosome 4 centromere	Oncor
D4Z1	digoxigenin	chromosome 4 centromere	Oncor
D6Z1	biotin	chromosome 6 centromere	Oncor
D6Z1	digoxigenin	chromosome 6 centromere	Oncor
D8Z1	biotin	chromosome 8 centromere	Oncor
D10Z1	biotin	chromosome 10 centromere	Oncor
D10Z1	digoxigenin	chromosome 10 centromere	Oncor
D13Z1/D21Z1	biotin	chromosome 13 and chromosome 21 centromere	Oncor
D14Z1/D22Z1	biotin	chromosome 14 and chromosome 22 centromere	Oncor
D17Z1	biotin	chromosome 17 centromere	Oncor
D17Z1	digoxigenin	chromosome 17 centromere	Oncor
D18Z1	biotin	chromosome 18 centromere	Oncor
D18Z1	digoxigenin	chromosome 18 centromere	Oncor
DXZ1	biotin	chromosome X centromere	Oncor
DXZ1	digoxigenin	chromosome X centromere	Oncor
Chromosome 4 wcp*	biotin	chromosome 4	Cambio
Chromosome 8 wcp*	biotin	chromosome 4	Cambio
m-*BCR/ABL* translocation probe	biotin /digoxigenin	m-*BCR* 22q11 *ABL* 9q34	Oncor
M- *BCR/ABL* translocation probe	biotin /digoxigenin	M-*BCR* 22q11 *ABL* 9q34	Oncor
YAC 13HH4	biotin	*MLL* 11q23	B.D. Young

*wcp = whole chromosome probe

2.2 METHODS

2.2.1 Sample preparation

Two types of samples were used in the study, samples remaining from cytogenetic investigation and samples separated on Ficoll by gradient centrifugation to obtain mononuclear cells (MNC) or high density fraction (HD). In all cases bone marrow had been taken for clinical assessment.

2.2.1.1 Samples remaining from cytogenetic investigation

Interphase and metaphase cells were obtained from cell pellets which had been prepared for chromosome analysis and stored in Carnoy's fixative (3:1 methanol/acetic acid) at -20°C for up to seven years. Cell pellets were resuspended in fresh fixative and slides were made. The slides were left to air dry overnight and subsequently used for FISH experiments. The additional slides were wrapped in aluminium foil and stored at -20°C. In some experiments previously G-banded slides which had been stored for up to three years in room temperature were used.

2.2.1.2 Samples prepared by gradient centrifugation

Bone marrow or peripheral blood samples were collected using heparin as a anticoagulant. The white blood cell count was performed on all samples in order to determine the approximate number of cells which would be recovered after separation.

2.2.1.2.1 Mononuclear (MNC) and high density (HD) cell isolation

Samples were diluted 50:50 with phosphate-buffered saline (PBS), if the WBC was low (i.e.<$2.0x10^9$/l) less diluent was added, more if it was high (>$500x10^9$/l). Approximately 10 mls of lymphoprep (Nycomed) was placed in a labeled universal and 10 mls of diluted bone marrow/peripheral blood was poured gently on top. Subsequently the sample was centrifuged at 800g (3000 rpm) for 20 minutes. Once the centrifugation was complete the mononuclear cell (MNC) layer was carefully removed using a glass pipette. The MNC layer was transferred to a second universal. Supernatant was removed from the first universal leaving the pellet containing the HD fraction. Approximately 15 mls of red cell lysis buffer (RCLB) was added to both universals. The pellet was resuspended and both universals left at room

temperature for 5 minutes. After lysis was completed, both universals were spun at 400g (1500 rpm) for 5 minutes. The supernatant was removed and the cells were washed in 20 mls of phosphate-buffered saline (PBS) by centrifugation at 400g (1500 rpm) for 5 minutes. The supernatant was discarded and cells were either transferred to cryovials and stored in liquid nitrogen or resuspended in PBS to a concentration of $1x10^9$/l prior to the preparation of the cytospins.

2.2.1.2.2 Liquid nitrogen storage

Freezing down

The cell pellet was put on ice. 0.5 mls of fetal calf serum (GIBCO, BRL) and 0.5 mls of 20% DMSO (SIGMA)/RPMI (GIBCO, BRL) was added to each cryovial. The cell pellet was left at -70°C overnight. The next day the cryovials were submerged in liquid nitrogen.

Thawing

When required, the cryovials were taken out of the liquid nitrogen container and warmed up to 37°C by agitating gently in a waterbath. The cell pellets were diluted with 5 mls of RPMI and spun down at 1000 rpm for 3 minutes. Cells were resuspended in fresh RPMI.

2.2.1.2.3 Preparation of cytospins

100 µl aliquots of cell suspension were spun at 400 rpm in a Cytocentrifuge (Shandon, UK) for 2 minutes. The cell distribution of the cells on a slide was checked using x40 objective lenses. If the cells were too crowded or too scanty, the cell concentration was adjusted. The slides were left to air dry overnight, wrapped in aluminium foil and stored at -20°C.

2.2.2 Identification of cell lineages

In order to investigate the clonal involvement of different cell lineages, bone marrow cells belonging to a particular cell lineage were first identified on cytospins by morphology using May-Grunwald-Giemsa (MGG) stain or by their immunophenotype using the alkaline-phosphatase anti-alkaline-phospatase (APAAP) technique. Subsequently, chromosomal abnormalities were investigated in the same preparations using the FISH technique (see below).

2.2.2.1 May Grunwald Giemsa (MGG) staining

Slides were stained with May Grunwald Giemsa (SIGMA) for 2 mins. Subsequently they were rinsed with distilled water and allowed to dry. After mounting in Apathys they were examined under the light microscope.

2.2.2.2 Alkaline-phosphatase anti-alkaline-phospatase (APAAP) technique

Slides stored in -20C in aluminium foil were allowed to defrost at room temperature for 20 minutes and then unwrapped and fixed in acetone for 10 minutes. They were washed in Tris buffered saline (TBS) for 3 minutes and transferred to a humidified box where diluted antibodies were applied. Slides were incubated for 30 minutes at 37 ^{o}C, washed in TBS for 2 minutes, followed by incubation with 20ul rabbit-anti-mouse (RAM) antibody (DAKO) diluted in 1/50 in TBS at 37 ^{o}C, washed again in TBS for 2 minutes, incubated with APAAP complexes (DAKO) for another 30 minutes at 37 ^{o}C and finally washed again in TBS for 2 minutes. For the amplification the same steps of incubation and washes were repeated but incubation time was decreased to 10 minutes. Alkaline phosphatase substrate (SIGMA) was made just prior to use and was filtered directly onto slides which were then left for 20 minutes in a humidified chamber. Subsequently slides were washed in TBS for 3 minutes, in tap water for 5 minutes and counterstained in haematoxylin (SIGMA) for 5 minutes. When the slides became dry they were mounted in Apathys. Slides were examined under the light microscope.

2.2.3 Fluorescence *in situ* hybridization (FISH)

2.2.3.1 Probe preparation

For most FISH experiments ready labeled probes were obtained from Oncor or Cambio. The investigation of lineage involvement of cases with 11q23 abnormalities was conducted using a YAC 13HH4 containing the *MLL* gene.

2.2.3.1.1 YAC culture

2% glucose/AHC cultures (50μg/ml of ampicillin (Sigma) and 7.5 ug/ml of tetracycline (Sigma)) were inoculated with yeast cells containing YAC 13HH4. Following incubation at 37^{0}C for 2 days, three of the four 5 ml cultures were inoculated into a 400ml AHC culture as above and incubated for a further 24 hours.

The remaining 5 ml culture was used to make 15% glycerol stocks which were stored at -70^{0}C.

2.2.3.1.2 Preparation of DNA

Following incubation, cells were centrifuged at 2500rpm for 15 minutes. The supernatant was removed and the pellet resuspended in 40ml of sterile distilled water. The resuspended pellet was transferred to a 50ml Falcon tube and centrifuged as above. The pellet was resuspended in 3.5ml of SCE and 85µl 2M DTT with 20 mg of yeast lytic enzyme (Sigma). The suspension was incubated at 37^{0}C for 90 minutes. The cell suspension was lysed by adding 7 ml of lysis buffer containing 100µg/ml proteinase K (Sigma). The lysate was heated to 65^{0}C for 15 minutes and cooled in water. A sucrose gradient was prepared using 11ml 20% sucrose followed by 11ml 15% sucrose and underlayed with 3 ml of 50% sucrose. The lysate was added carefully to the top of the gradient and then spun at 26,000rpm in a Beckmann centrifuge for three hours at 20^{0}C. The upper 30ml of the sucrose gradient was aspirated. The DNA layer was removed with a 10ml wide-bore pipette and placed in a universal. A dialysis bag was washed through with distilled water, clamped at one end and the DNA poured into the other end and clamped. The DNA was dialysed overnight against 2 liters pH 7.5 TE (10 mM Tris/0.1 Mm EDTA) at 4^{0}C.

The DNA was transferred from the dialysis bag into a falcon tube and equal volumes of butanol added. Following mixing and quickly centrifuging at 12,000g in a microfuge for 20 seconds the upper phase was discarded and the process repeated until less than 5ml of DNA suspension was left. The DNA was transferred to an eppendorf containing 100% ethanol and sodium acetate (10% v/v). Following centrifugation for 5 minutes in a microfuge and removal of the ethanol, the precipitated DNA was vacuum dried for 10-15 minutes and the pellet resuspended in 50 µl of TE.

2.2.3.1.3 Gel electrophoresis

The DNA concentration was estimated by gel electrophoresis. For this a 0.8% agarose gel was prepared containing 0.5µg/ml of ethidium bromide as below: 0.56g of powdered agarose (Sigma) was mixed with 70ml of 0.5 x TBE buffer (0.045*M* Tris-borate, 0.001*M* EDTA) and heated until the agarose dissolved. The solution was cooled to 60^{0}C prior to the addition of ethidium bromide. The agarose/ethidium bromide solution was poured onto a prepared gel. The gel was left to dry at room

temperature. Five microlitres of each DNA sample were mixed with 2µl of gel-loading buffer (bromophenol blue) and loaded into each well in the gel. A series of standard DNA solutions were prepared by mixing known concentrations of uncut lambda DNA (20, 50, 100, 200, 400ng/µl)(Boehringer) with 2µl of gel loading buffer. The gel was run at 5V/cm. The gel was photographed using short wavelength ultraviolet irradiation and the quantity of DNA in the sample estimated by comparing the sample DNA with the standards.

2.2.3.1.4 Probe labeling

To a 1.5ml eppendorf tube containing 1µg of probe DNA 5µl 10x nick translation buffer (0.5*M* Tris-HCl, 50*mM* $MgCl_2$, 0.5mg/ml BSA, 5µl dithiothreitol (DTT) (0.1*M*), 4µl of nucleotide mix (0.5*mM,* dATP, dGTP, dCTP, 0.1*mM* dTTP) (Boehringer), 1µl of biotin, 2µl of DNA polymerase I (10U/L) (Boehringer) and 5µl DNase I (Sigma) (1:1000 of 1mg/ml solution) was added. The contents were mixed gently and incubated in a waterbath at 15^0C for two hours. Five microlitres of 0.5*M* EDTA (pH 8) was added to stop the reaction.

2.2.3.1.5 Fragment size

Five microlitres of probe DNA were mixed with 2 µl of bromophenol blue and 5 µl of ddH_20 and loaded onto the gel. The DNA of ϕX174 was loaded as a reference ladder. The gel was run at 5V/cm for approximately 90 minutes.

2.2.3.1.6 Probe preparation and precipitation

To 1µg of labeled probe, 50µg of Human-Cot-1 DNA (Gibco) and 100µg of salmon sperm DNA (Sigma) was added. This was followed by 1/10th the volume of 3M sodium acetate, pH 5.2 and 2.5 times 100% ethanol. The suspension was mixed, vortexed in a microfuge and incubated at -70^0C for 30 minutes. Following centrifugation in a microcentrifuge for 15 minutes the supernatant was discarded and the pellets washed in 70% ethanol and centrifuged for 5 minutes. The supernatant was discarded and the pellet lyophilized for 10 minutes. The dried pellet was resuspended in hybridization buffer (50% deionized formamide (Cambridge BioScience), 2x SSC, 10% dextran sulfate (Sigma) and stored at -20^0C until required.

2.2.3.2 Slide pretreatment

Coverslips from G-banded slides were removed by soaking in Carnoy's fixative. Removal of DPX mounting medium and Giemsa stain was achieved by washing in three changes of fresh Carnoy's fixative. Unbanded slides containing the cytogenetic preparation and cytospin slides which had been stored in -20°C were taken out of the freezer and left at room temperature to defrost. After 20 minutes the aluminum foil was removed. Slides containing the cytogenetic preparations were incubated at 37°C in 2x SSC for 30 minutes. Cytospin slides were fixed in 100% methanol prior to investigation by FISH. All slides were put through an ethanol gradient (70%,90%, 100%).

2.2.3.3 Denaturation

DNA on the slides was denatured by incubation in 70% deionized formamide/2 x SSC on a hotplate at 72°C for 2 minutes. After denaturation the slides were dehydrated through an ethanol gradient (70%, 90%, 100%). Prior to denaturation, alpha satellite centromeric probes were diluted with hybrisol VI (Oncor, Gaithesburg MD) to a concentration of 1ug/1ul. Alpha-satellite chromosome-specific probes, whole chromosome probes (WCPs) and the YAC probe were denatured by heating at 72°C for 5 minutes. WCPs were pre-annealed at 37°C for 30 minutes prior to hybridization. The m-*BCR-ABL* and M-*BCR-ABL* translocation probes did not require denaturation.

2.2.3.4 Hybridization

The probe was placed on the slide and covered with a glass coverslip and incubated overnight in a humidified chamber at 37°C. Approximately 100ng of WCP, 50ng of centromeric probe and 500ng of YAC were applied per hybridization.

2.2.3.5 Post hybridization washes

The YAC probe, WCPs and the m-*BCR-ABL* and M-*BCR-ABL* translocation probes were washed 0.5xSSC at 70°C. The alpha-satellite, chromosome-specific probes were washed in 0.5xSSC at 72°C.

2.2.3.6 Detection

After washes were completed FITC-avidin (or FITC-avidin and rhodamine-labelled anti-digoxigeneine mixture in double/triple colour experiments) (Oncor, Gaithesburg MD) was applied, slides were covered with plastic coverslips and incubated for 20 minutes at 37°C. Washes were done in 0.05% tween 20/ 4xSSC three times (2 minutes each). After washing anti-avidin antibodies (Oncor, Gaithesburg MD) were applied and slides were incubated at 37 °C for 20 minutes. Washes and the final layer of FITC-avidin (or FITC-avidin and rhodamine-labelled anti-digoxigeneine mixture in double/triple colour experiments) (Oncor, Gaithesburg MD) were done as previously described. After the final wash, propidium iodide (detection with FITC) or DAPI (detection with rhodamine) (Vector Laboratories, Burlingame, CA) at a concentration of 80ng/ml counterstain was applied.

2.2.3.7 Triple colour FISH

Some FISH experiments required that three chromosomal gains be detected simultaneously. For these, four probes were used to obtain three colours as follows. Probes labelled with digoxigenin (dig) were detected with rhodamine labeled anti-digoxigenin antibodies (red). Biotin (bio) labelled probes were detected with fluorescein labelled avidin (FITC) (green). To obtain a third colour (yellow) dig and bio labelled probes were mixed in a 1:1 ratio.

2.2.3.8 Scoring

Control Samples

Three 'chromosomally normal' bone marrow or peripheral blood samples per probe were used as controls. For each control 1000 (unless stated otherwise) nuclei were scored with each probe. The mean (M) and standard deviation (SD) for control cells with a given pattern of hybridization signals (e.g. one additional signal or *BCR-ABL* fusion signal) were calculated for each probe.

Patient Samples

For each case at least 300 nuclei were scored per probe unless stated otherwise. Cells which were overlapping or had poor or ambiguous signals were excluded. The number of cells having a given pattern of hybridization signals (e.g. one additional signal or *BCR-ABL* fusion signal) was recorded. If the number of cells with this pattern was

greater than the mean control value + twice the standard deviation (M+2SD) a clone was presumed to be present.

2.2.4 Microscopy

Slides were examined with a Zeiss-Axioskop. Images were taken with the laser confocal microscope (Olympus BH2) (early experiments) or with Zeiss Axioskop coupled with a CCD camera and with software from Digital Scientific, Cambridge, UK.

Chapter 3

HYPERDIPLOIDY IN NORMAL AND FAILED ALLs

3.1 Summary

High hyperdiploid (>50 chromosomes) and low hyperdiploid (47-50 chromosomes) clones in the leukaemic cells at diagnosis of acute lymphoblastic leukaemia (ALL) identify patients with a good and relatively good prognosis respectively. In the cytogenetic laboratory at the Royal Free Hospital, cytogenetic analysis in ALL fails (no clone detected in a yield of <20 metaphases) in 26% of cases and yields only normal metaphases in 12% of the successful cases. In this chapter patterns of chromosomal gains in hyperdiploid clones have been used to look for hyperdiploidy in patients with ALL, where cytogenetics had failed (35 cases, 13 adults, 22 children), or yielded only normal metaphases (30 cases, 13 adults, 17 children). Fluorescence *in situ* hybridization (FISH) with alpha-satellite, chromosome-specific probes was applied to interphase nuclei, at diagnosis, targetting chromosomes X, 6, 16, 18, 20 and 21. FISH analysis revealed 5 cases with high hyperdiploidy, two cases with low hyperdiploidy and two cases with unspecified high or low hyperdiploidy.

A high hyperdiploid clone was detected by FISH in 3/22 (9.1%) children with failed cytogenetics which was lower than the 31% detected by cytogenetics in this age group. No case with a high hyperdiploid clone was found amongst children classified as normal. In adults, high hyperdiploidy was detected by FISH in 1/13 (7.7%) failed cases and in 2/13 (15.4%) normal cases. These figures compare with 12% detected by cytogenetics. The sizes of clones indicated by interphase FISH in cases classified as normal (seen in adults only) were lower (range 10%-42%) than in failed cases (range 68%-82%) (seen in children and adults). The present study suggests that failure to detect a high hyperdiploid clone by metaphase cytogenetics may be more frequent in adults than in children. Clones smaller than 50% have been shown to occur in adult ALL and these appear to evade detection even when 20 cells have been analysed

3.2 Introduction

The stratification of patients with acute lymphoblastic leukaemia (ALL) according to prognosis is important in therapy planning. The important prognostic implications of chromosome ploidy at diagnosis, initially reported by Secker-Walker at al. (Secker-Walker et al., 1978), are now well established (IWCL3, 1981a; Swansbury et al., 1981; Kaneko et al., 1982; Secker-Walker et al., 1982b; Williams et al., 1982; Bloomfield et al., 1986; Pui et al., 1987; Michael et al., 1988; Pui et al., 1988; Van der Plas et al., 1992b; GFCH, 1993; Raimondi, 1993; Kobayashi et al., 1994). Hyperdiploidy (clones with chromosomal gain) is important because it is mostly associated with a good prognosis. There are three hyperdiploid groups, low hyperdiploidy (47-50 chromosomes), high hyperdiploidy (50-65 chromosomes) and triploidy or near tetraploidy (>68 chromosomes).

A significant proportion (23%-33%) of ALL patients escape classification by yielding insufficient dividing cells in bone marrow samples for chromosome analysis (Jackson et al., 1990; Secker-Walker, 1990; Harris et al., 1992). Furthermore, up to 39% of successfully analysed cases have only chromosomally normal metaphases or have only a single (random) cell with chromosomal gain or structural change or fewer than three cells with the same chromosomal loss, which are insufficient for the abnormality to be classified as clonal (Walters et al., 1990). Since standard cytogenetic analysis of 20 metaphases is unlikely to detect clones present in less than 11% of dividing cells (Hook, 1977), patients classified as 'normal' may have minor or inert clones.

Fluorescence *in situ* hybridization (FISH) can be used to detect chromosomal gains in the bone marrow of patients with haematological malignancy (Jenkins et al., 1992; Romana et al., 1993). In contrast to cytogenetics, FISH allows for the investigation of non-dividing as well as dividing cells.

Moorman et al. (Moorman et al., 1996) observed patterns of chromosomal gain in patients with hyperdiploid ALL. Gains of certain chromosomes or certain combinations of chromosomes were often seen in one hyperdiploid group (e.g. high hyperdiploid) but rarely, if at all in another (e.g. low hyperdiploid). He proposed a strategy for the use of FISH in interphase cells whereby, using ten chromosome specific probes in a maximum of three steps, cases could be successfully recognised as being hyperdiploid and assigned to one of three ploidy groups: low hyperdiploid, high

hyperdiploid and triploid/tetraploid. In this chapter the possibility that high hyperdiploid clones may be found in the diagnostic bone marrow samples from patients with ALL whose cytogenetics was normal or failed is examined.

3.3 Materials and methods

3.3.1 Patients

In the cytogenetics laboratory at the Royal Free Hospital, between 1990 and 1996, 414 patients were diagnosed with acute lymphoblastic leukaemia of which 263 were classified as abnormal, 36 as normal and 107 as failed. Bone marrow samples were available from 65 patients in whom the diagnostic cytogenetics was either normal or had failed. Bone marrows from three healthy, chromosomally normal individuals were used as controls.

3.3.2 Strategy

The strategy used to identify hyperdiploid cases and assign them to a particular ploidy group was proposed by Moorman et al. (Moorman et al., 1996) and is illustrated in figure 3.1. The strategy was based on the observation that certain chromosomes or combinations of chromosomes are often gained together in one ploidy group but rarely, if at all, in another. It was proposed that FISH employing a total of ten chromosome-specific probes in a step wise fashion would identify hyperdiploid cases and assign them as low hyperdiploid, high hyperdiploid or triploid/tetraploid. The purpose of this study was primarily to identify high hyperdiploid cases, so that only a part of the above strategy was adopted (see fig 3.1 for the part of the strategy used in the study). The strategy requires that cases be initially screened with probes to chromosomes X and 18 (see fig 3.1). Depending on the result of the initial screening, cases are then investigated as follows: cases positive for both X and 18 are investigated with probes to chromosomes 16 and 20, cases positive for either X or 18 are investigated with probes to chromosomes 6 and 21 (see fig 3.1). Cases negative for both probes to chromosomes X and 18 are presumed not to contain a high hyperdiploid clone and need not be investigated further. The results are then interpreted as follows: cases positive for X, 18, 16 and 20 are assigned as triploid/tetraploid, cases positive for X, 18 and either 16 or 20 are assigned as high hyperdiploid, cases positive for either X or 18

and both 6 and 21 are assigned as high hyperdiploid, cases positive for either X or 18 and negative for both 6 and 21 are assigned as low hyperdiploid (see fig 3.1). Cases positive for X and negative for 18 and positive for either 6 or 21 are investigated with the probe to chromosome 14. Cases positive for 14 are then assigned as high hyperdiploid, those negative for 14 are assigned as low hyperdiploid (see fig 3.1). Cases positive for 18 but negative for X and positive for either 6 or 21 are considered to be hyperdiploid but cannot be specified (unspecified) whether high, low or triploid/tetraploid (see fig 3.1).

3.3.3 Fluorescence *in situ* hybridization

Probes

Chromosome-specific alpha-satellite probes (Oncor, Gaithesburg) for chromosomes X, 6, 16 and 18 were available for this study. Also used were probes to chromosomes 14 and 21 that cross hybridize with chromosome 22 and 13 respectively.

Probe hybridization, detection and scoring

are described elsewhere (section 2.2.3)

Control values

Control values for trisomic cells were calculated as described (section 2.2.3).

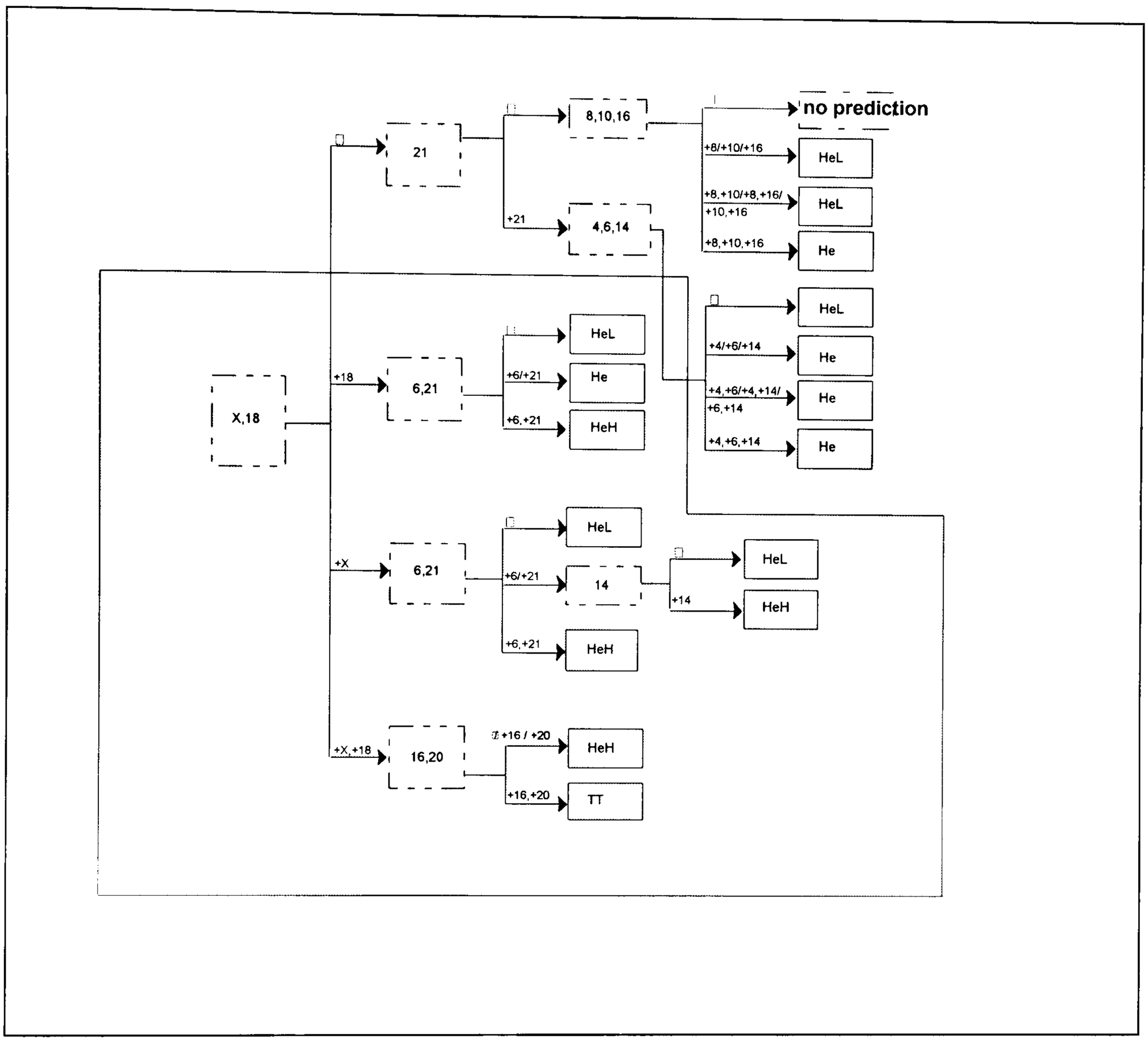
3.4 Results

3.4.1 Cytogenetic Results at Diagnosis at the RFH

Laboratory records of diagnostic cytogenetics in ALL showed that no abnormal clone was found in approximately a third of cases. This was either due to cytogenetic failure (26%) or to a classification of normal (9%) (fig 3.2). Among successfully analysed cases approximately 34% had a hyperdiploid clone with low hyperdiploid 9%, high hyperdiploid 20% and triploid/tetraploid 5% (fig 3.3). If children (<15 years) and adults (≥15 years) were considered separately the incidence of all hyperdiploid groups in children was greater (42%) than that in adults (28%). This was mostly due to a greatly increased incidence of high hyperdiploidy in childhood (31%) compared with that (12%) in adults (fig 3.4-3.5).

Fig 3.1

The strategy for the use of chromosome specific probes with interphase FISH to identify hyperdiploid cases and assign them to low, high and triploid/tetraploid subgroups as proposed by Moorman et al (Moorman et al., 1996).



Key

The probes used are shown in broken boxes, the final classification in solid boxes. He hyperdiploid (unspecified), HeL low hyperdiploid, HeH high hyperdiploid, TT triploid/tetraploid. The part of the strategy which was used in this study is indicated by the small frame.

Fig 3.2

The distribution of patients between chromosomal categories normal, abnormal and failed cytogenetics. 414 patients with ALL investigated at diagnosis at the RFH over a 6 year period (1990-1996)

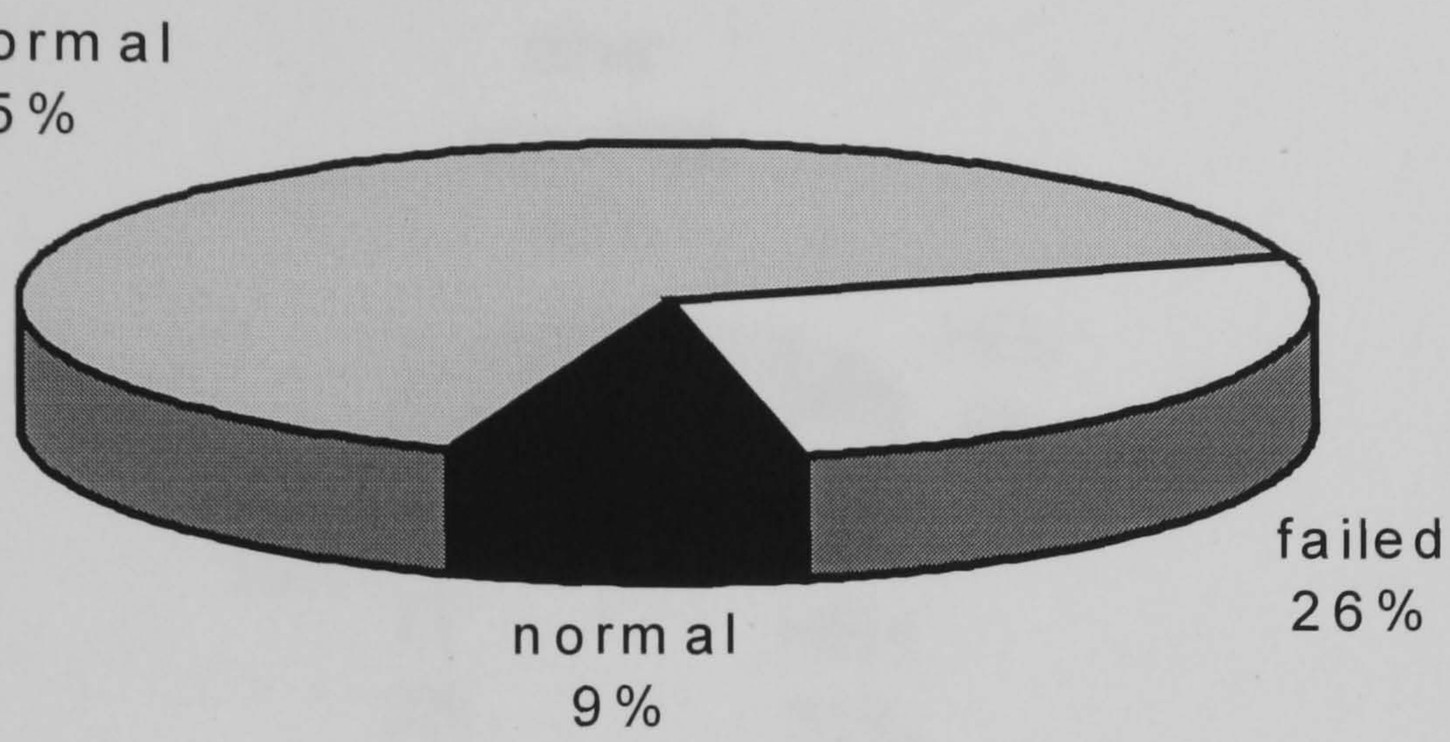


Fig 3.3

The distribution of patients between chromosomal categories high hyperdiploidy (HEH), low hyperdiploid (HEL), triploid/tetraploid (TT), normal and other abnormal. 299 patients with ALL and successful cytogenetics investigated at diagnosis at the RFH over 6 year period (1990-1996)

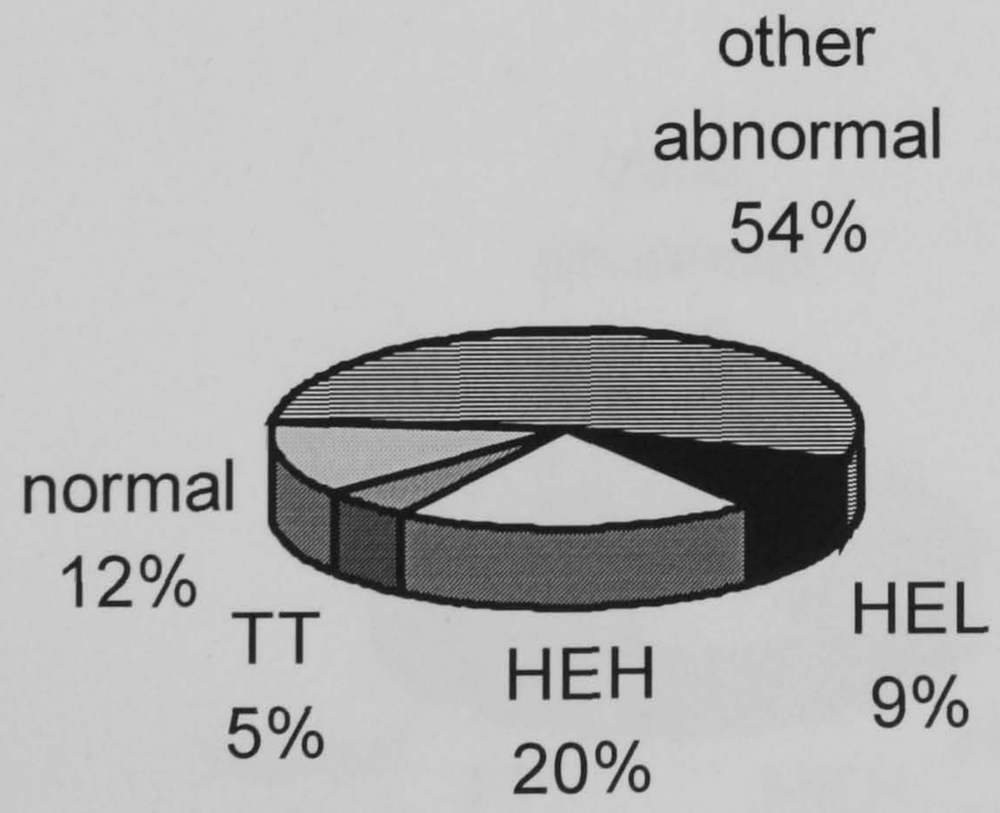


Fig 3.4

The incidence of different chromosomal subgroups amongst 125 children with ALL, successfully karyotyped at diagnosis, investigated at the RFH between 1990-1996

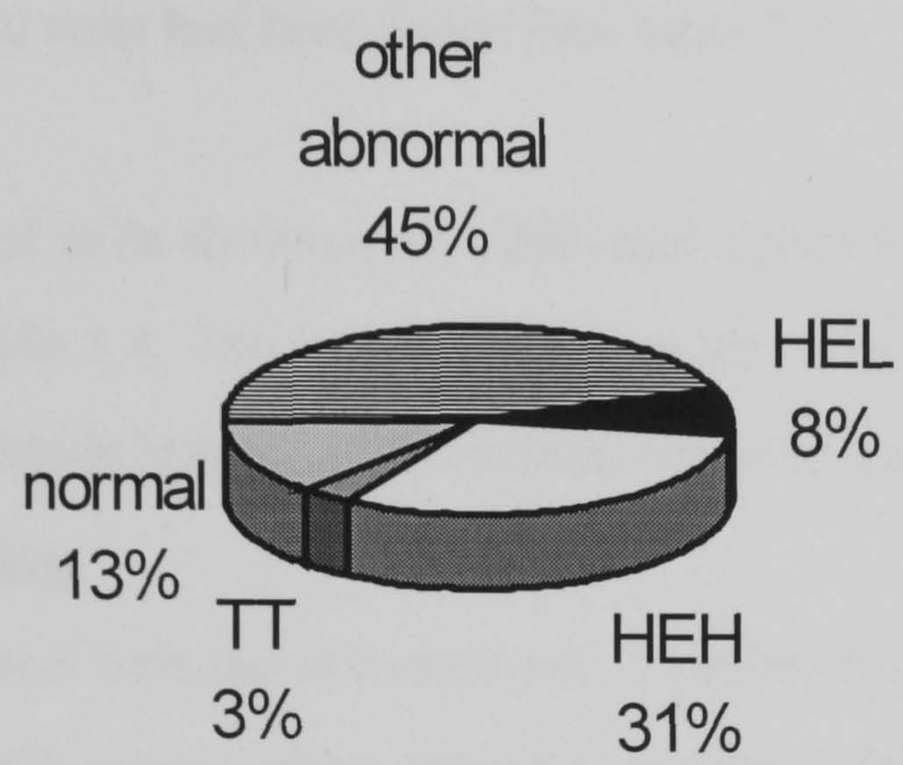
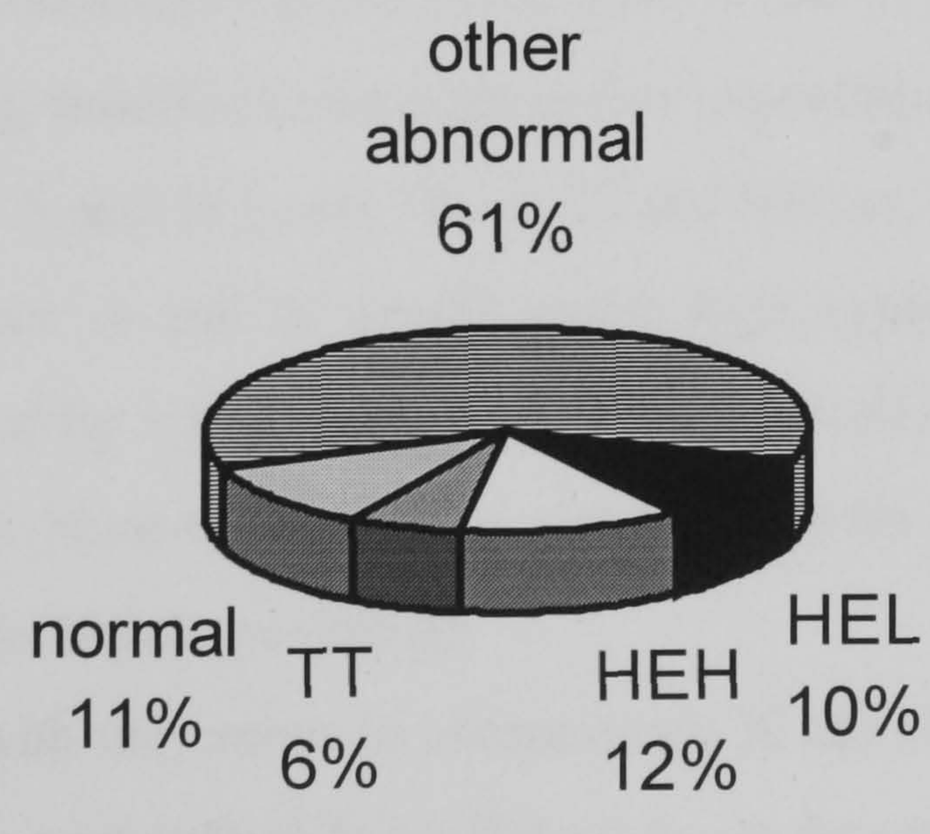


Fig 3.5

The incidence of different chromosomal subgroups amongst 174 adults with ALL, successfully karyotyped at diagnosis, investigated at the RFH between 1990-1996



3.4.2 Investigation of hyperdiploidy by FISH

Patients

Clinical features, immunological data and cytogenetics of 65 patients investigated by FISH is shown in tables 3.1 and 3.2. In 35 patients cytogenetics had failed (cases 1-35) (table 3.1) and in 29 (cases 36-65) had been normal (table 3.2). In 11 cases one or more single (nonclonal) abnormal cells had been found (see table 3.3).

Controls

Control values (M+2SD) of cells showing an additional signal for the probes used in the study are shown in table 3.4. The lowest value was seen for probe 18 (1.4%) and was highest for the probe which hybridizes to chromosomes 13 and 21 (3.5%).

Interphase FISH investigation

All 65 cases were hybridized with the chromosome X probe. Nine cases (cases 1, 17, 18, 27, 34, 35, 53, 56 and 57) were positive for +X (see table 3.5) and were assigned as hyperdiploid (see strategy, section 3.3.2). Cases 1 and 35 lacked material for further analysis. The remaining 63 cases were then hybridized with the chromosome 18 probe. Four (cases 17, 18, 27 and 56) were positive for +18 (see table 3.5). Further probing with probes 6 and 21 (see section 3.3.2 and fig 3.1) in the 3 cases positive for X but not 18 (cases 34, 53 and 57) revealed 1 case (case 53) with significant numbers of positive cells with both probe 6 and probe 13/21 identifying therefore this case as high hyperdiploid (see table 3.5). In the remaining two cases (case 34 and 57) both probings proved to be negative identifying therefore those cases as low hyperdiploid.

Cases positive with both probes X and 18 (cases 17, 18, 27 and 56) (see table 3.5) were subsequently probed with probes 16 and 20 to distinguish high hyperdiploidy from tetraploidy (see section 3.3.2 and fig 3.1). Cases 27 and 56 were positive for 16, cases 17 and 18 were positive for 20. None of those cases were positive for both probes so that those cases were assigned as high hyperdiploid.

Fifty six cases were negative with the probes to chromosome X and chromosome 18 probe and were presumed not to contain high hyperdiploid clones (see strategy, section 3.3.2).

Table 3.1
Clinical data on patients with acute lymphoblastic leukaemia and 'failed' cytogenetic analysis

case no	age (yrs)/ sex	immunophenotype	WBC $x10^9/l$	cytogenetics N [Ab]
1	2/M	c-ALL	31.9	4
2	2/M	c-ALL	8.8	1
3	2/M	c-ALL	8.5	0
4	3/F	c-ALL	15.2	3
5	3/F	Nk	6	0
6	3/F	Nk	5.7	16
7	3/F	Nk	Nk	8
8	4/F	Nk	10	10
9	4/M	Nk	43	1
10	4/M	T-cell	50.3	0
11	4/M	c-ALL	33.5	0
12	5/M	pre-B	27	17
13	5/M	Nk	254	1
14	6/F	Nk	488	12
15	6/M	Nk	5.3	12
16	7/M	T-cell	189.6	6[1]*
17	8/M	c-ALL	2.3	0
18	9/M	c-ALL	13.5	0
19	10/M	c-ALL	3.8	5
20	10/M	Null	21.7	17[1]*
21	13/M	Nk	71.3	6
22	14/F	c-ALL	157	18
23	15/F	c-ALL	Nk	3
24	15/M	c-ALL	5	1[1]*
25	16/M	T-cell	171	4
26	19/M	T-cell	37	6[1]*
27	21/F	pre-B	Nk	1[1]*
28	21/M	c-ALL	55.4	0
29	25/F	c-ALL	3.8	12
30	30/F	T-cell	2.4	1
31	31/M	Nk	23	$4[3^{@}]$*
32	32/F	Null	Nk	1
33	37/F	Nk	Nk	0
34	41/M	pre-B	59.3	0
35	64/F	c-ALL	4	0

* see table 3.3, @= non clonal, N= number of normal cells, [Ab] = number of abnormal cells

Table 3.2

Clinical data on patients with acute lymphoblastic leukaemia and 'normal' cytogenetic analysis

case no	age (yrs)/ sex	immunophenotype	WBC $x10^9/l$	cytogenetics N [Ab]
36	1/F	c-ALL	6.9	20
37	1/M	Nk	Nk	20
38	2/F	c-ALL	46	20
39	2/F	c-ALL	13.5	20[1]*
40	2/M	Nk	Nk	20[1]*
41	3/F	c-ALL	12.9	20
42	4/F	c-ALL/pre-B	10.3	22
43	4/F	Nk	7.1	20
44	4/F	Nk	7.1	20
45	5/M	Nk	Nk	20
46	5/M	c-ALL	3.4	20
47	6/F	Nk	21	20
48	6/M	c-ALL	Nk	21
49	7/F	Nk	Nk	20
50	12/M	c-ALL	1.2	20
51	13/F	c-ALL	64.3	20
52	14/M	T-cell	Nk	20
53	16/F	c-ALL	0.6	29[1]*
54	16/M	Nk	74	20
55	17/F	Nk	Nk	20
56	17/M	pre-B	Nk	20[1]*
57	22/M	T-cell	Nk	1[1]*
58	23/F	T-cell	43	30
59	24/M	c-ALL	1.7	29
60	27/M	Nk	Nk	20
61	31/F	Nk	3	20
62	34/F	c-ALL	8.1	20
63	43/M	T-cell	60	31
64	44/M	T-cell	4.8	20
65	65/M	B-cell	22.5	20

* see table 3.3, @= non clonal, N= number of normal cells, [Ab] = number of abnormal cells

Table 3.3
Karyotypes of cases with single (random) abnormal cells.

case no	karyotype
16	46,XY[16] / 47,XY,+10[1]
20	46,XY[10] / 46,XY,del(5)(p15)[1]
24	46,XY[1] / 47,XY +mar[1]
26	46,XY[5] / 46,XY-9-14 + 2mars[1]
27	58 [inc] [1]
31	46,XY[11] / 48,XY,+22,+mar[1] / 45,XY,add(8)(p),-11/12[1] / 46,XY,t(5;9)(q31;q34)[1]
39	46,XX[19] / 46,XX,?del(9)(p22)[1]
40	46,XY[20] / 89,XXYY [1]
53	46,XX [20] / 52,XX,+X,+4,+6,+8,+17,+22 [1]
56	46,XY [20] / 57,XY, +A,+B,+C,+D,+G [1]
57	46,XY[20] / 50, XY,-1,-3,-4,+6,+6,+7,+8,-11,+12,+13,+17,-18,+20,+22[1]

Table 3.4
Control values based on the mean and standard deviation of the number of cells showing an additional signal for each probe used in the study

chromosome specific probe	Mean (%)	SD (%)	control values (M+2xSD) (%)
X males	1.2	0.4	2
X females	1.1	0.4	1.9
6	0.9	0.3	1.5
21*	2.5	0.5	3.5
16	1.3	0.3	1.9
18	1	0.2	1.4
20	1.4	0.2	1.8

Key
* cross hybridizes with chromosome 13
SD = standard deviation
M = mean

Table 3.5

Patients classified as hyperdiploid by FISH.
The percentage of cells with an additional signal to the probes tested

case no	probes						clone detected
	X	18	6	21	16	20	
1	68.2*	NMA	NMA	NMA	NMA	NMA	HE
17	85.7*	81.0*	NI	NI	1.8	76.9*	HEH
18	72.4*	78.0*	NI	NI	1.7	75.8*	HEH
27	75.6*	60.9*	NI	NI	73.9*	1.5	HEH
34	78.7*	1.2	1.4	2.9	NI	NI	HEL
35	82.3*	NMA	NMA	NMA	NMA	NMA	HE
53	47.1*	1.1	38.1*	39.5*	NI	NI	HEH
56	9.7*	7.9*	NI	NI	11.0*	1.7	HEH
57	41.1*	1.3	1.3	3.2	NI	NI	HEL

KEY
* significantly above control values
HE hyperdiploid >46 chromosomes
HEH high hyperdiploid 50+ chromosomes
HEL low hyperdiploid 47-49 chromosomes
NI not investigated
NMA no material available

In summary, FISH analysis revealed 5 cases with high hyperdiploidy (cases 17, 18, 27, 53 and 56), two cases with low hyperdiploidy (cases 34 and 57) and two cases with unspecified subgroup of hyperdiploidy (cases 1 and 35). High hyperdiploidy was found in 2/22 children (cases 17 and 18) and in 1/17 adults (case 27) with failed cytogenetics (see table 3.1) and in 2/13 adults (cases 53 and 56) with normal cytogenetics (see table 3.2).

The incidence of high hyperdiploidy detected by FISH in cytogenetically failed and normal cases is compared in fig 3.6. The incidence of high hyperdiploidy in children with failed cytogenetics detected by FISH was 9.1%. FISH failed to detect any cases with high hyperdiploidy among children with normal cytogenetics. Among adults, FISH detected 7.7% with high hyperdiploidy among failed and 15.4% among normal cases. This was considerably less than that detected cytogenetically in children in our laboratory (31%). The incidence of high hyperdiploidy detected by FISH compared

with that detected by cytogenetics (12%) in adults was lower among failed cases (7.7%) and greater among normal cases (15.4%).

The size of the clone indicated by interphase FISH in cases originally classified as normal (cases 53, 56, 57) was lower (range 10% to 42%) than that in cases classified as failed (cases 1,17, 18, 27, 34, 35) (range 68% to 82%) (see fig 3.7).

The three adults (cases 27, 53 and 56) found to be high hyperdiploid by FISH, had each shown a single high hyperdiploid metaphase on cytogenetic investigation (see table 3.3). Full analysis had been obtained for one of these cells. In case 53 the cytogenetic result showed, +X,+4,+6,+8,+17,+22. FISH confirmed +X,+6 but also showed +21 suggesting either that the clone included more trisomies than seen in the single cell or that analysis of the single cell was incorrect.

Fig 3.6

The incidence of high hyperdiploidy detected by FISH in 30 cases with normal and 35 cases with failed cytogenetics compared with that detected cytogenetically in 299 successfully analyzed patients.

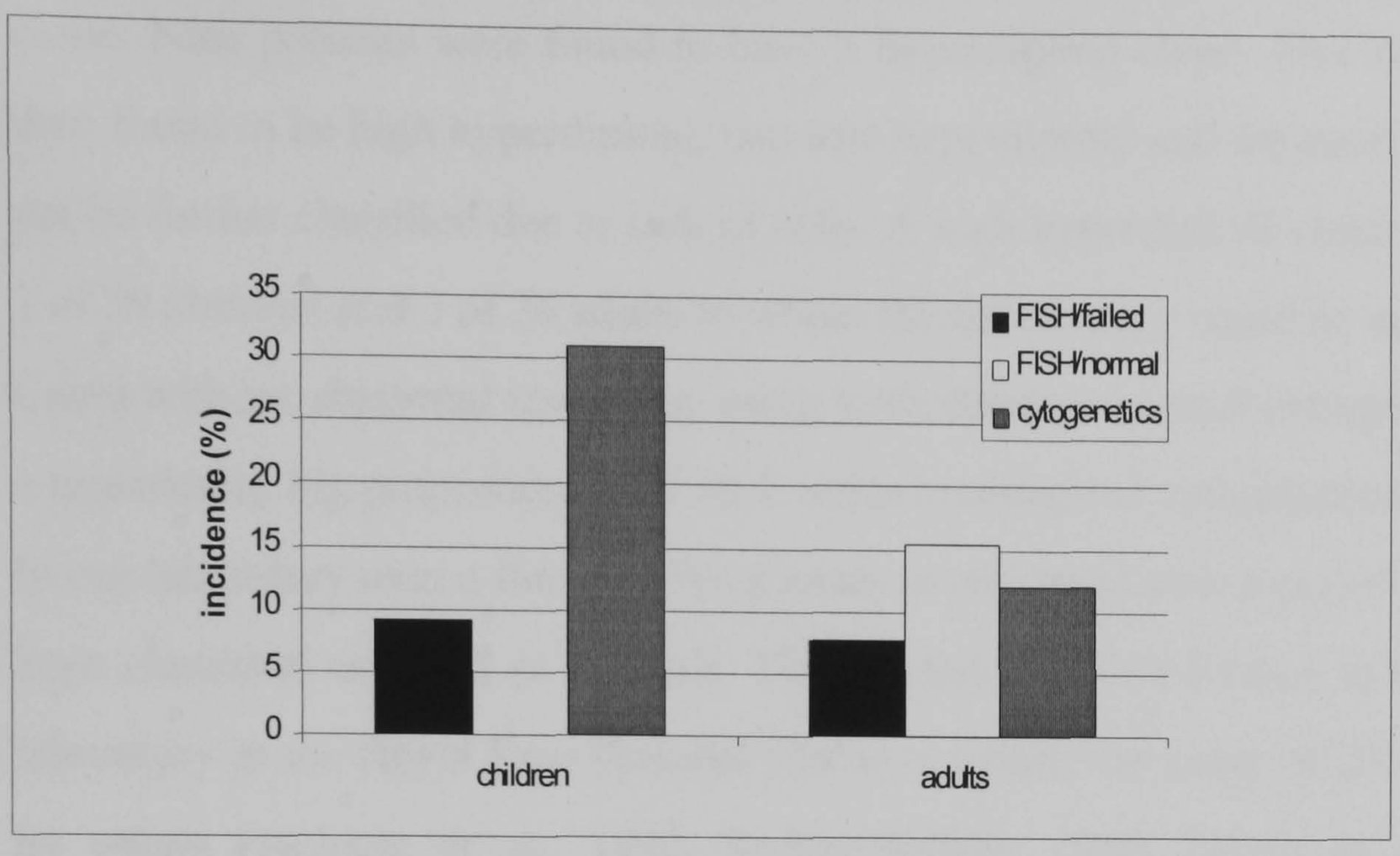
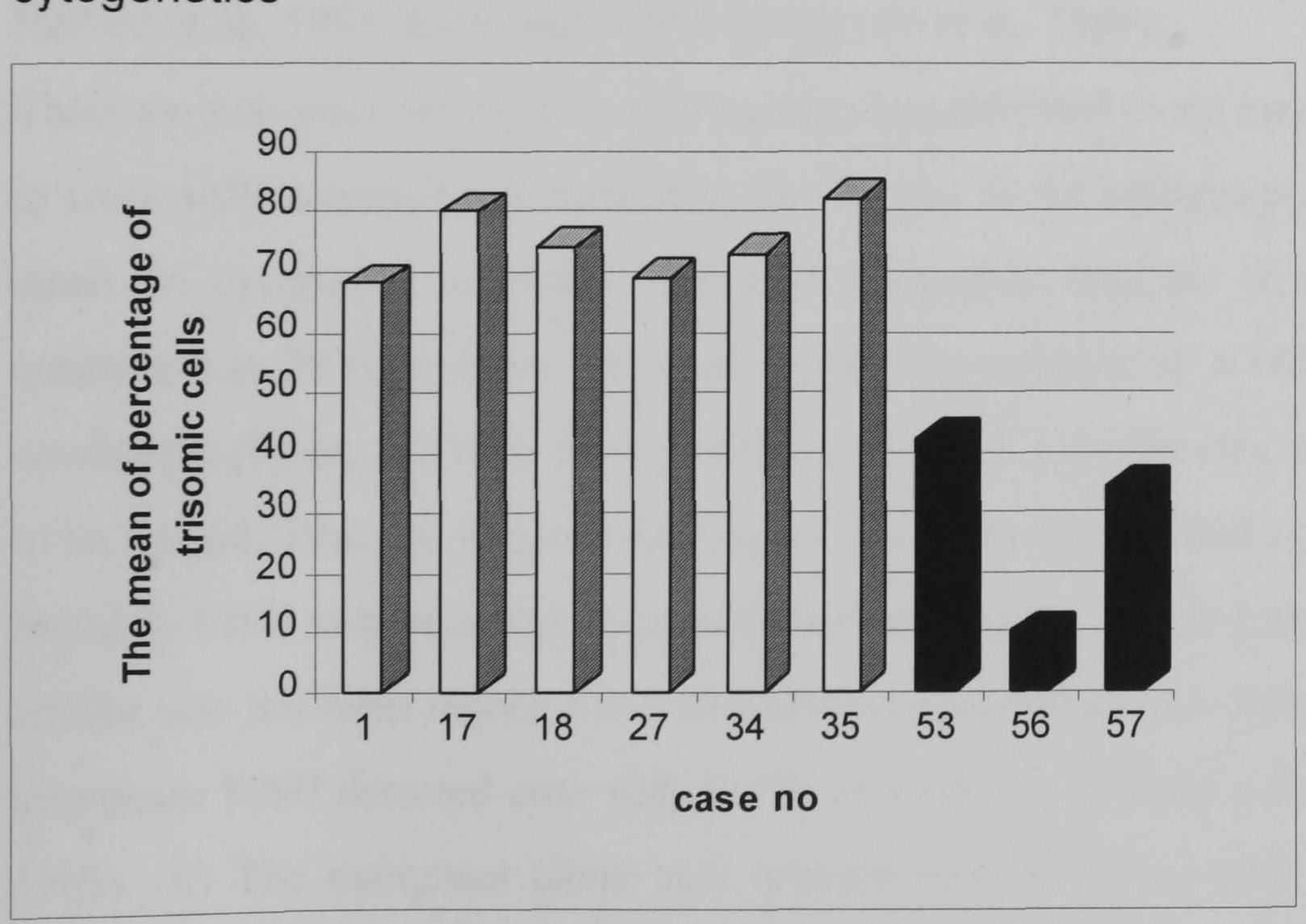


Fig 3.7

The sizes of the hyperdiploid clones as indicated by the percentage of trisomic cells detected by FISH in patients with normal and failed cytogenetics



□ cases with failed cytogenetics ■ cases with normal cytogenetics

3.5 Discussion and conclusions

Sixty five patients with ALL for whom cytogenetic analysis had failed or yielded only normal cells have been investigated for the possible presence of a high hyperdiploid clone. Nine patients were found to have a hyperdiploid clone. Five out of nine were then found to be high hyperdiploid, two low hyperdiploid and the remaining two could not be further classified due to lack of cells. A high hyperdiploid clone was detected in 2 of 39 children and 3 of 26 adults to whom the full strategy could be applied.

Cases without abnormal clones i.e. cases with failed or normal cytogenetics constitute a remarkably big proportion of all ALL cases investigated cytogenetically at diagnosis. In our laboratory over a third (35%) of cases investigated over a period of 6 years have been classified as failed or normals. The incidence of failed cases in the cytogenetics laboratory at the Royal Free Hospital (26%) is within the range of 23%-33% reported by others (Jackson et al., 1990; Secker-Walker, 1990; Harris et al., 1992). The incidence of chromosomally normal cases among all successfully investigated cases in our laboratory (12%) is at the lower end of the range of 9% to 39% reported by others (Fletcher et al., 1989; Walters et al., 1990; Van der Plas et al., 1992b; GFCH, 1993; Harbott et al., 1993; Raimondi, 1993; Kobayashi et al., 1994).

There are two possible ways in which a high hyperdiploid clone may escape detection in cases with successful cytogenetics. (i) The size of the malignant clone may be too small for cytogenetic detection. Standard cytogenetic analysis is restricted by time constraints to 20 metaphases. This will exclude the presence of a 11% clone with 95% confidence (Hook, 1977). It follows that any clone of a similar size or smaller is likely to be missed. This was illustrated by one of our cases with normal cytogenetics. It was found by FISH to have a high hyperdiploid clone of 10%. A high hyperdiploid clone of similar size has been reported in a chromosomally normal ALL relapse. In one report interphase FISH detected case with 13.5% of high hyperdiploid cells (Heerema et al., 1993). (ii) The malignant clone may become inert in culture and in spite of being relatively abundant, the chromosomally abnormal cells may be under represented among the dividing cells. This was illustrated by the two remaining cases with normal cytogenetics which were found to have 34% and 42% of hyperdiploid cells. This finding suggests that, in a proportion of high hyperdiploid cases, malignant cells divide

in culture less efficiently than their normal counterparts leading to apparently small or inert clones. This finding is supported by the report by Manabe et al. (Manabe et al., 1992). He described a serum -free assay to compare survival requirements of leukaemic B-cell progenitors. In this assay the only two cases not giving rise to viable cells in culture were patients with high hyperdiploid clones.

The incidence of high hyperdiploidy among children by FISH was 9.1% in failed and 0% in normals. This was much lower than the 31% of cases with high hyperdiploidy detected in children with successful cytogenetics in our laboratory and reported by others (range 19.6% to 33%) (Fletcher et al., 1989; Van der Plas et al., 1992b; GFCH, 1993; Harbott et al., 1993; Raimondi, 1993; Kobayashi et al., 1994). These findings may suggest that high hyperdiploid cases of childhood ALL are less likely to fail in culture. The incidence of high hyperdiploidy detected by FISH among adults was 7.7% in failed and 15.4% in normals. These figures are closer to the 12% of high hyperdiploidy among adult cases with ALL detected by cytogenetics in our laboratory and to the numbers reported by others 7.0% (Groupe Francais de Cytogenetique Hematologique, 1996) and 10% (Walters et al., 1990; Secker-Walker et al., 1997). This finding suggests that cytogenetic analysis is neither more nor less likely to fail in adult ALL with high hyperdiploidy than in any other group.

Interestingly, while the relative proportion of children and adults with high hyperdiploidy detected by FISH among failed cases mirrored that detected by cytogenetics (more children than adults were high hyperdiploid), the proportion of childhood and adult cases in normals was reversed. This finding suggests that high hyperdiploid clones in adults are more likely to become inert than those in children.

In three out of five cases found to be high hyperdiploid by FISH, the initial cytogenetic investigation had revealed a single high hyperdiploid metaphase. This shows that finding of a single high hyperdiploid cell in the diagnostic bone marrow of patients with ALL may be suggestive of the presence of a high hyperdiploid clone.

In summary, the data presented here, suggest that failure to detect a high hyperdiploid clone by metaphase cytogenetics may be more frequent in adults than in children. This study demonstrates that clones smaller than 50% may evade detection even when 20 cells have been analysed. In conclusion interphase FISH provides a useful adjunct to cytogenetics for the detection of high hyperdiploid clones in ALL.

Chapter 4

SINGLE-CELL TRISOMY

4.1 Summary

Finding a chromosomally abnormal clone in the bone marrow of a patient with a haematological disorder is important to confirm the neoplastic nature of the disease. The presence of a particular chromosomal abnormality may be indicative of prognosis. The presence of a clone can only be confirmed when two or more cells with the same structural change or chromosomal gain are detected. A bone marrow sample which yields 20 metaphases of which no two are clonal is classified as normal. Since cytogenetic analysis detects only actively dividing clones, the presence of a single (random) abnormal cell among 20 cells analysed raises doubts about its clonal nature. Fluorescence *in situ* hybridization (FISH) enables rapid detection of certain chromosomal abnormalities in both metaphase and interphase cells thus enabling the detection of minor or inactive clones. Seven samples each from a patient with haematological malignancy at diagnosis or in remission and each having one or more random abnormal cells were investigated, thus: The six diagnostic samples were from patients with AML (3 cases) and a cell with +4 (1 case) +7 (1 case) or two cells with +9,+22 /+10,+17,+17 (1 case); from patients with MDS (three cases) and a cell with +8 (2 cases) or +9 (one case). The seventh sample was from a patient with ALL in remission and had one cell with +4. One of the patients, a male aged 66 years, with MDS (refractory anaemia with ringed sideroblasts) was found to have a minor clone with trisomy 8 in his diagnostic marrow. A follow-up marrow 42 months later showed no trisomy 8 cell among 62 metaphases analyzed, and the percentage of trisomic cells using FISH on interphase cells was within the control range. This patient has survived for more than 42 months requiring no treatment. The single cell abnormalities in six cases proved to be random events. Thus it appears that single cell abnormalities may not be clonal or may indicate the presence of a minor clone well below the level of cytogenetic detection. The prognostic significance of such minor clones is at present unclear.

4.2 Introduction

Cases in which a clone is not detected in the bone marrow are described as chromosomally normal. These cases are of two kinds: those in which no abnormal cell is detected and those with random changes i.e. having a single cell showing structural change or chromosomal gain, or no more than two cells with the same chromosomal loss. Among many possible reasons why, in some cases, clones are not found may be the limitations of the cytogenetic technique itself which is restricted to dividing cells, and limited by time constraints to analysis of no more than 20 cells. Cases which are prone to misclassification are those where the size of a clone is small and where neoplastic cells are not dividing.

Whole chromosome gain resulting in trisomy of one chromosome is a feature of clonal change in all haematological malignancies at diagnosis (United Kingdom Cancer Cytogenetics Group, 1992). Established subgroups in AML include those with trisomies 4, 8, 9, 11, 13, 21 or 22 (Walker et al., 1994); in MDS those with trisomies 7, 8, 9 (Noel et al., 1993). In ALL trisomies 8, 10, 16, 21 or X account for rare subgroups (Secker-Walker, 1990; Secker-Walker, 1994). Remission in the acute leukaemias is associated with loss of the cytogenetically abnormal clone. However, single abnormal cells are occasionally found in remission marrows (Heerema et al., 1993).

Fluorescence *in situ* hybridization (FISH) using whole-chromosome or alpha-satellite centromeric probes is a technique which can be used to detect the presence of trisomies in both mitotically active and resting cells. It also makes it possible to include large numbers of cells in the analysis, making for more sensitive clone detection.

In this chapter, samples of bone marrow, each having a single abnormal cell, were investigated by FISH using chromosome paints on metaphase cells and alpha satellite centromeric probes on interphase cells to confirm the trisomy and to discover whether the single cell trisomies were truly random events or whether they represented minor or inactive clones.

4.3 Materials and methods

4.3.1 Patients

The patients investigated by FISH each had one or two non-clonal cells with chromosomal gain (or gains) and a haematological malignancy as follows: acute myeloid leukaemia (AML) (3 patients at diagnosis), myelodysplastic syndrome (MDS) (3 patients at diagnosis) and acute lymphoblastic leukaemia (ALL) (1 patient in haematological remission). Bone marrows from three haematologically healthy individuals were used as controls.

4.3.2 Fluorescence *in situ* hybridization

Samples investigated

Metaphase spreads were investigated on slides which had been prepared for chromosome analysis (case 2 and 3). They had been G-banded and stored at room temperature for 1 and 3 years.

Interphase cells were obtained from cell pellets which had been prepared for chromosome analysis and stored in Carnoy's fixative (3:1 methanol : acetic acid) at -20°C for up to three years (cases 1-7). Cell pellets were resuspended in fresh fixative and slides were made immediately prior to FISH.

Probes

Hybridization to metaphase spreads was carried out using a whole chromosome probe (wcp). Biotin labelled whole chromosome probes for chromosomes 4 and 8 (Cambio, Cambridge) were used.

For interphase FISH, biotin labelled alpha satellite centromeric probes, specific for the centromeres of chromosomes 4, 7, 8, 9, 10, 17 (Oncor,Gaithesburg MD) were used.

Hybridization, detection and interphase scoring

For the details of hybridization, detection and interphase scoring refer to section 2.2.3.

4.4 Results

4.4.1 Cytogenetic results of diagnostic and remission samples at RFH between 1985 and 1994.

Table 4.1 shows the total number of samples investigated from patients with AML or MDS at diagnosis and patients with ALL in remission in the cytogenetic laboratory, The Royal Free Hospital School of Medicine, between April 1985 and March 1994 as follows: those with an abnormal clone, those with a clone of 47 chromosomes due to a single trisomy, and those with one or two (random) abnormal cell(s) in cases lacking a clone.

Table 4.1

The incidence of clonal trisomy and of single trisomic cells in cytogenetically normal bone marrow in patients with AML and MDS at diagnosis and ALL in remission among samples karyotyped over a 9 year period in the cytogenetic laboratory at the RFHSM

diag	stage	total no	chromosomally abnormal cases				chromosomally normal cases						
			total no	with a clonal trisomy			total no	with a single trisomic cell					
				+4	+8	+9		+3	+4	+8	+9	+17	+22
AML	Diag	260	156	2	8	1	104	1	1	0	0	1	0
MDS	Diag	134	57	0	4	0	77	0	1	3	1	0	1
ALL*	Rem	296	5	0	1	0	291	0	1	0	0	0	1

* Ph+ cases excluded

4.4.2 FISH investigation of patients with single abnormal cell

Patients

The clinical and cytogenetic data of each of the 7 patients investigated by FISH are given in Table 4.2. Three patients had AML (cases 1-3), three had MDS (cases 4-6) and one had ALL. The random abnormal cell or cells had been seen at diagnosis (cases 1-6) or in remission (case 7). Case 1 had two different abnormal cells, with a combination of trisomy 9 and 22 and a combination of trisomy 10 with tetrasomy 17.

In cases 2 and 4 although the trisomy was identified, the quality of banding was less than optimal. For these cases the G-banded slide was destained and hybridized with whole chromosome probes (wcp) to chromosome 4 (case 2) or wcp to chromosome 8 (case 4). In each case the suspected trisomy was confirmed (Fig 4.1 & 4.2). In two cases the trisomies were found in metaphases with chromosome loss (cases 3 & 4). Case 6 had tetrasomy 8 in a high hyperdiploid cell with 58 chromosomes. Follow-up samples were obtained for cytogenetic analysis from case 1, four and five months later and from case 5, fifteen and twenty nine months from diagnosis. No similar trisomic cell had been found in either case in 10 or 20 cells analysed. Case 7 (investigated in remission) had a high hyperdiploid karyotype at diagnosis which did not include trisomy 4. Previous and subsequent remission samples of case 7 had been chromosomally normal on 35-50 cells analysed.

Table 4.2

Diagnosis, Age, sex, survival and cytogenetics of patients with single cell abnormalities at diagnosis

case no	diagnosis	age(yrs) /sex	event-free survival months	cytogenetics
1	AML M4	9/M	7[1]	46,XY [18]/48,XY,+9,+22 [1]/ 49,XY,+10,+17,+17 [1]
2	AML M0	26/M	18	46,XY,22p+c [18]/47, idem,+4 [1]
3	AML	65/M	NA	46,XY [15]/45,XY,+7,-10,-18 [1]
4	MDS RARS	65/M	48+	46,XY[29]/40,X-Y,-6,+8,-11,-15,-17,-17,-20 [1]
5	MDS	74/F	42	46,XX [19]/47,XX,+9 [1]
6	MDS RARS	90/F	76+	46,XX[15]/58,XX+X,+8,+8,+9,+12,+12,+13, +13, +14,+16,+18,+21 [1]
7[2]	ALL[3]	8/M	40+	46,XY [40]/47,XY,+4 [1]

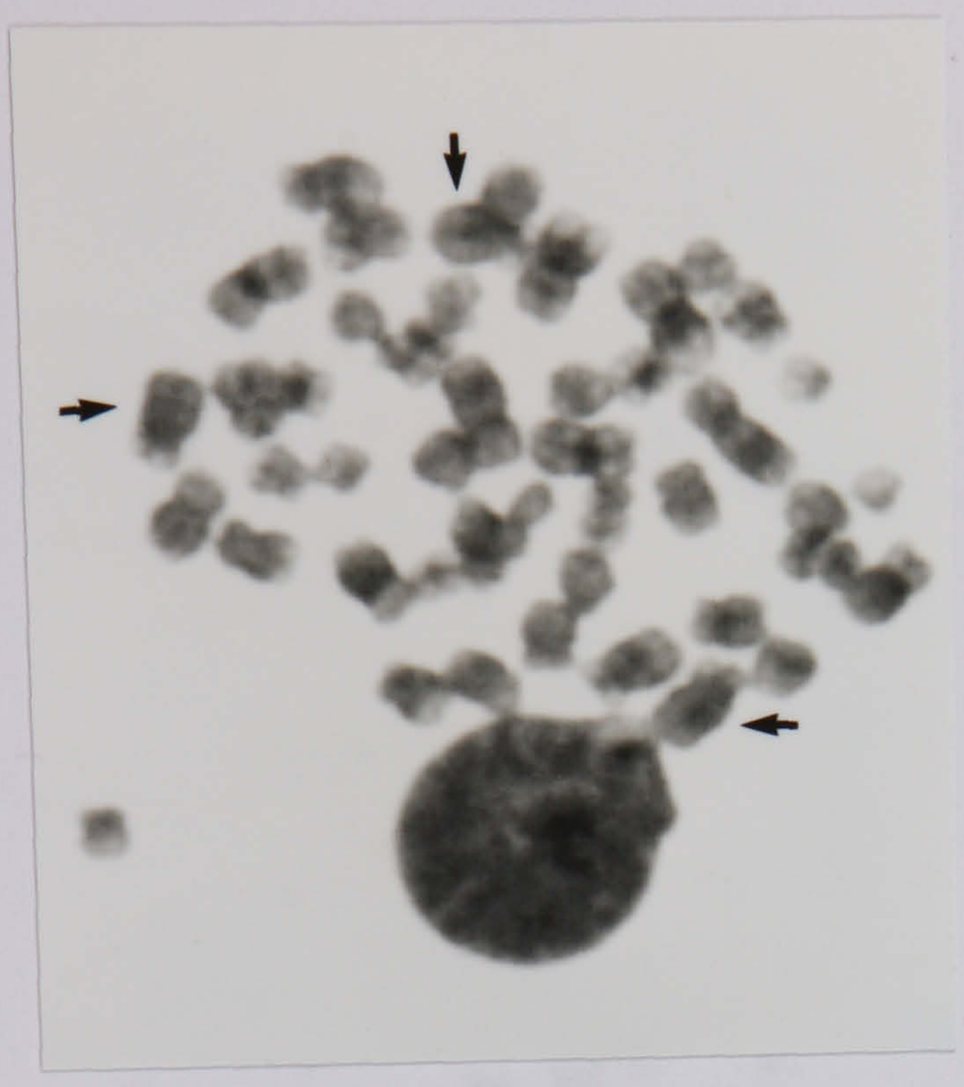
[1] = Died following bone marrow transplantation in first complete remission.
[2] = In remission 7 weeks from diagnosis.
[3] = Clonal karyotype at diagnosis 51,XY,+X,+6,+14,+21,+21 [19] /46,XY [1]
AML = Acute myeloid leukaemia,
MDS = Myelodysplastic syndrome,
ALL = Acute lymphoblastic leukaemia
RARS = Refractory anaemia with ringed sideroblasts, NA = not available

Fig 4.1

FISH investigation in case 2.

A

Metaphase spread stained with G-banding. Note three chromosomes 4 (arrowed).



B

The same metaphase spread hybridized with whole chromosome probe (wcp) to chromosome 4.

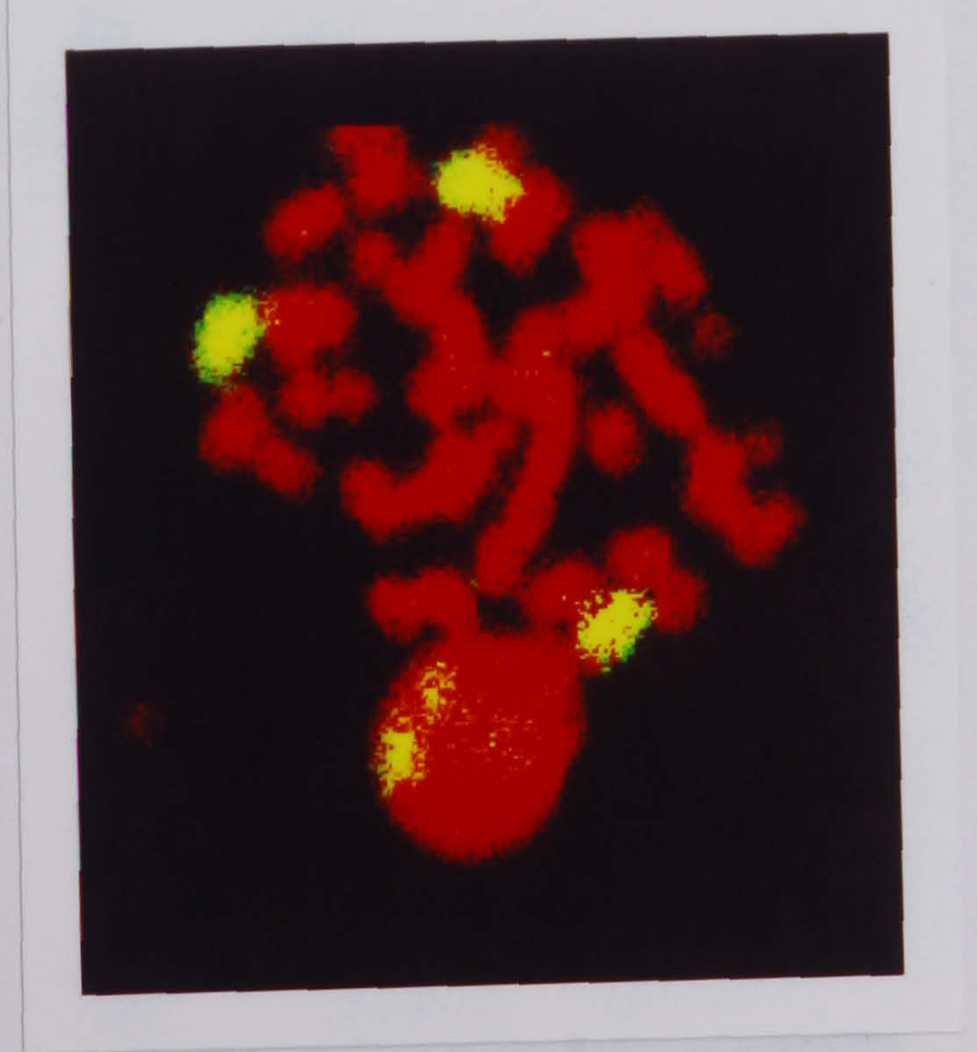
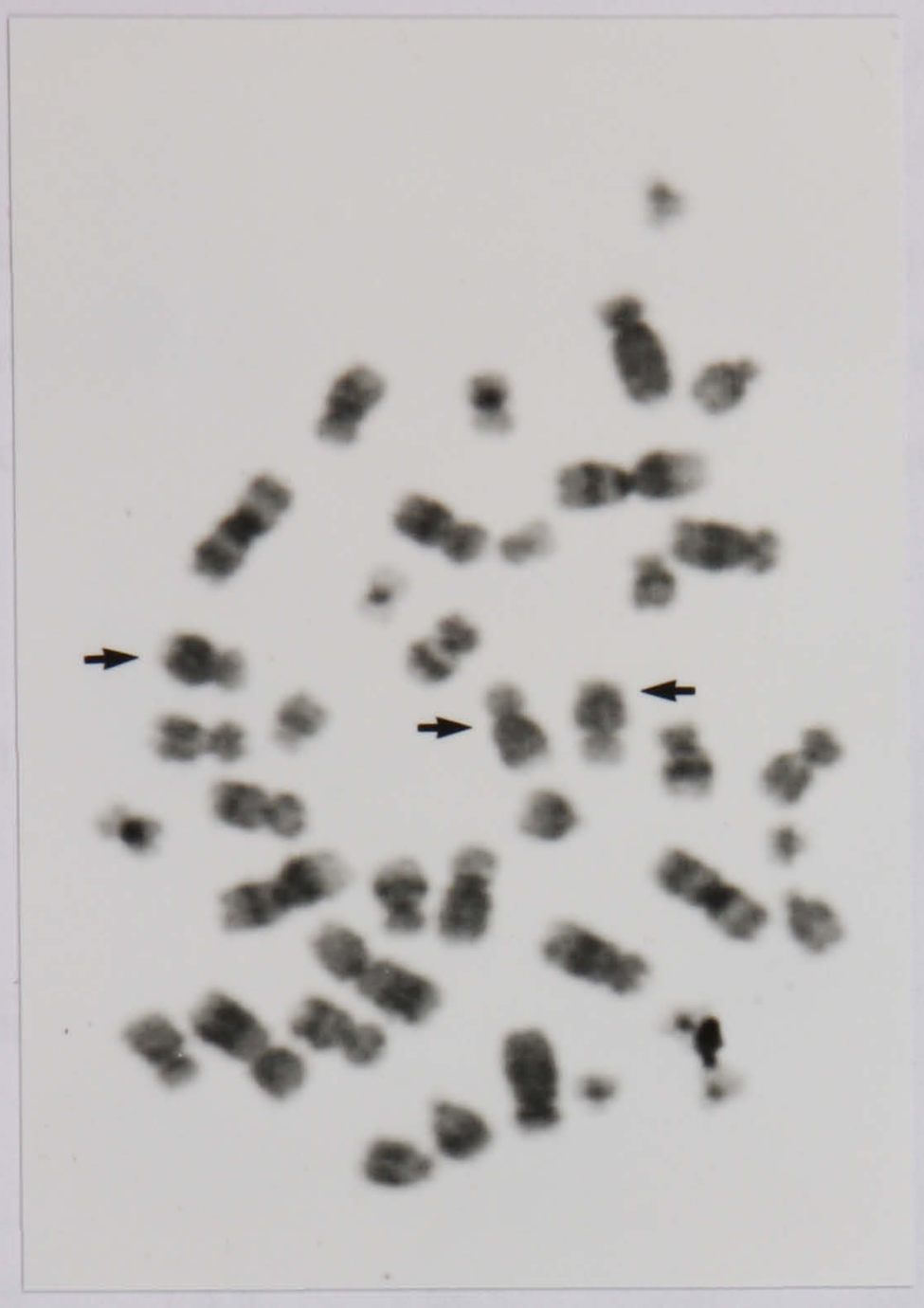
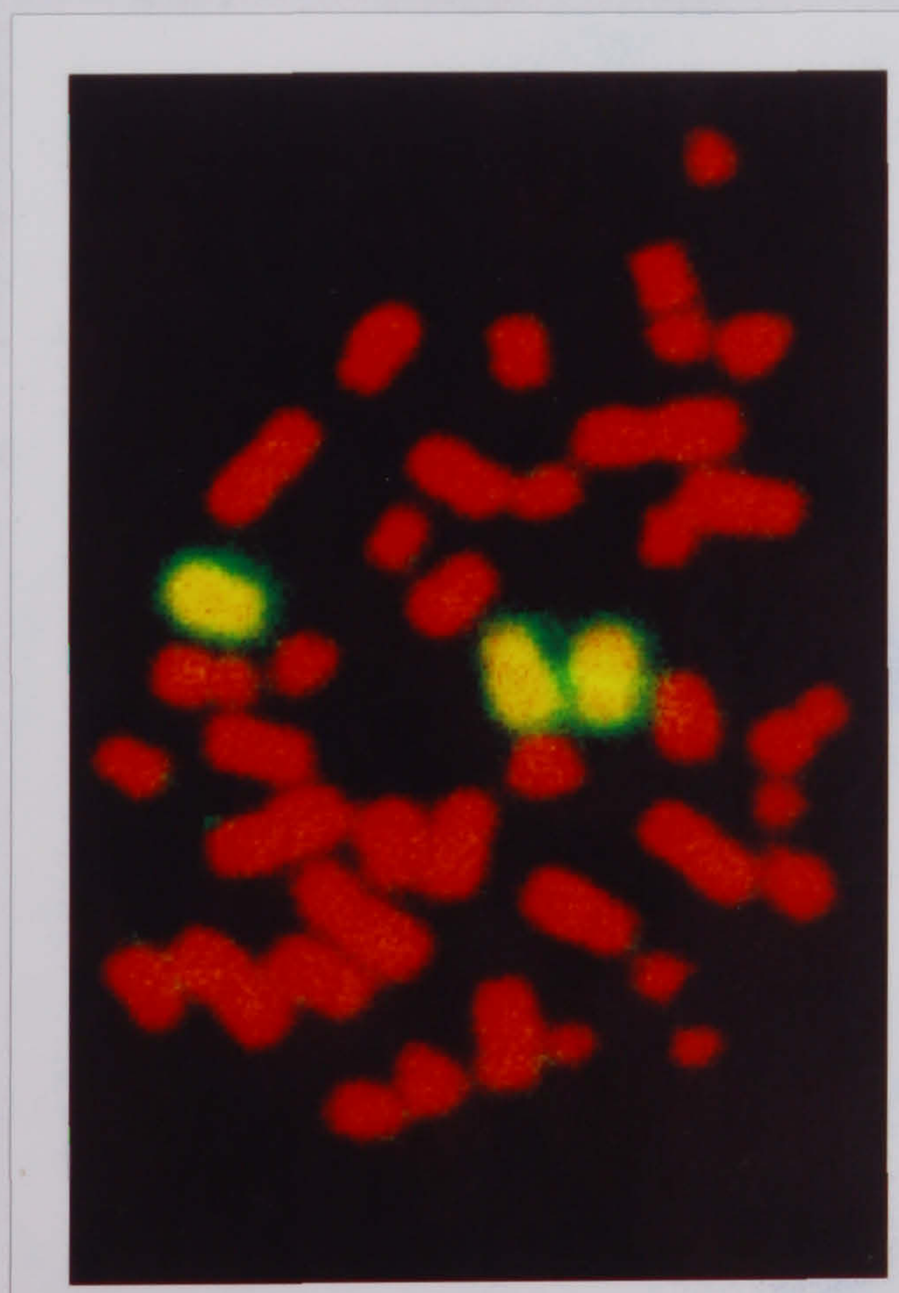


Fig 4.2
FISH investigation in case 4.
A
Metaphase spread stained with G-banding. Note three chromosomes 8 (arrowed).



B
The same metaphase spread hybridized with whole chromosome probe (wcp) to chromosome 8.



Interphase Fluorescence *in situ* hybridization

The percentages of cells with 0-4 signals is given for bone samples of patients 1-7 in table 4.3. They are compared with control values (M+2SD) for cells with three signals obtained for each probe.

Out of seven patients examined only case 4 showed a significant number of trisomic cells at diagnosis, with 1.5% trisomy 8 cells which was just greater than the control value for the probe (1.06%). Examination of a follow up sample 42 months later showed the percentage of trisomic cells by FISH within the control range. No trisomic cell was found cytogenetically among 62 metaphases examined.

Table 4.3

Investigation of 1000 cells by FISH analysis of bone marrow samples from cases 1-7 with a single cell trisomy identified cytogenetically

case	probe	percentage of cells with 0-4 signals					Control values (M+2xSD) for cells with 3^ signals
		0	1	2	3	4	
1	10	0.7	2.3	95.2	1.8	0.0	2.0+0.3
1	17	0.6	7.7	90.4	1.2	0.2	0.2+0.2
1	9	0.5	4.7	94.0	0.6	0.2	0.8+0.6
2	4	0.9	2.9	94.8	1.3	0.1	1.6+0.4
3	7	3.9	3.7	90.0	2.2	0.2	1.9+0.4
4	8	0.5	3.2	94.5	1.5	0.3	0.9+0.2
4*	8	0.6	2.9	95.6	0.8	0.1	0.9+0.2
5	9	0.4	4.2	95.0	0.4	0.0	0.8+0.6
6	8	1.4	2.4	95.1	0.9	0.2	0.2+0.3
7	4	1.0	1.8	95.6	1.5	0.1	1.6+0.4

^ 4 signals in cases 1 (chromosome 17-specific probe) and case 6

* 42 months from diagnosis

4.5 Discussion and conclusions

Seven patients with a haematological malignancy and one or two cells with apparently random trisomies at diagnosis or in remission have been investigated for possible clonality. All single cell trisomies examined in our study had been unequivocally verified either by banding on well spread metaphases or, in two cases, supplemented by chromosome painting. Clonality was demonstrated in only one case, a patient with MDS was found to have a small clone with trisomy 8.

Trisomy 8 is the most frequent trisomy in human neoplasia and has been shown to account for 194/349 of all cases with a single chromosomal gain as the only change (Heim, Mitelman, 1986). Trisomy 8 is the most frequent numerical change in MDS and is found in 19% of all cases (Mufti, 1992). In the cytogenetic database at the RFHSM MDS cases showed trisomy 8 as the sole change in 4/57 or 7% of chromosomally abnormal cases. These 4 cases each had a major clone involving >60% of the cells analysed. In addition a single cell with trisomy 8 was found in 3/77 or 4% of chromosomally normal cases. The present study demonstrated that one of the two cases available for study had a small clone.

The FISH technique employed in this study is not only capable of verifying clonality but also determines whether the cytogenetic absence of a clone was due to its small size or to the lack of division of the clonal cells. The clone detected in this study turned out to be very small, and was only 0.44% above control values. Although the clone size was very small it is worth remembering that it represents between 10^{10}-10^{11} of total cell mass (Van Bekkum, 1984). This size of clone is similar to that detected by FISH in other studies with single cell trisomy 8 in at least 20 metaphases analysed. Clonality has been demonstrated in 14/23 such cases investigated, predominantly in cases of MDS (Jenkins et al., 1992; Chen et al., 1993b; Kibbelaar et al., 1993) (see table 4.4). In four cases the size of the clone was equal to control values (mean +2SD) (Kibbelaar et al., 1993), other cases showed clones of between 0.4% and 8% (median 1.6%) above control values (Jenkins et al., 1992; Chen et al., 1993b; Kibbelaar et al., 1993) (see table 4.4). The picture emerges that when a single cell with trisomy 8 is found in MDS it is the size of the clone rather than the inability of the clone to undergo cell division that is responsible for the apparent lack of a clone on cytogenetic analysis.

Table 4.4

Reported cases with single trisomy 8 cell detected by cytogenetics and analyzed by FISH.

case no	no of cells analyzed on GTG banding	percentage of interphase cells with 3 signals (FISH)	controls Mean +2xSD (FISH)	reference
1	36	3.0	3.0	Kibbelaar et al, 1993
2	30	3.0	3.0	Kibbelaar et al, 1993
3	20	4.0	3.0	Kibbelaar et al, 1993
4	30	3.0	3.0	Kibbelaar et al, 1993
5	20	3.0	3.0	Kibbelaar et al, 1993
6	21	2.4	1.0	Chen et al, 1993
7	20	2.4	1.0	Chen et al, 1993
8	20	7.0	1.0	Chen et al, 1993
9	20	2.2	1.0	Chen et al, 1993
10	30	3.0	2.0	Jenkins et al, 1993

In MDS, patients with a clonal cytogenetic abnormality have a worse prognosis than those without and trisomy 8 has been shown to be associated with a particularly poor prognosis (Nowell, Besa, 1989). The significance and clinical importance of small clones in the bone marrow of patients with MDS is difficult to evaluate at present. The patient with a minor clone at diagnosis described in this study has responded in a manner more reminiscent of patients without clonal involvement. He remained well for 42 months, is currently off all treatment and requires only occasional blood transfusions. In addition the lack of trisomic cells detected by FISH 42 months later indicates that such minor clones may be transitory.

It cannot be ruled out that the clone is falsely represented in the bone marrow due to sampling in cases where the leukaemic population is not evenly distributed. However if the clone is indeed present in a small minority of cells it may indicate that chromosomal change is a late event which occurs after clonal expansion. Alternatively it may represent an early stage of the disease when the clone is maintained but has not yet suffered a second hit required to confer a true proliferative advantage over normal cells.

Other trisomies of chromosome 4, 7, 8, 9 & 17 were shown to be random events or, if clonal, to be below the level of detection of this technique. This contrasts with a report

by Chen et al (Chen et al., 1993b) who used interphase FISH to investigate the clonality of single trisomic cells in six cases of MDS, two of AML and one each of myeloproliferative disorder and chronic lymphocytic leukaemia. In each case the presence of a clone was confirmed.

One patient with AML had a single cell with trisomy 4. This was the only AML with a random trisomy 4 cell found in the cytogenetic laboratory at the RFHSM (see table 4.1). In the cytogenetic laboratory at the RFHSM single trisomic cells in chromosomally normal cases of AML have been rare with only three examples in 104 (3%) normal cases analysed (see table 4.1). Trisomy 4 is well established as a clonal change, particularly in AML M2 and M4 (Prieto et al., 1987; Sandberg et al., 1987; Suenaga et al., 1993). It is however a rare subgroup in AML, accounting for 1.3% of all abnormal cases (see table 4.1).

Another patient had a single cell with trisomy 7. No case of AML with clonal trisomy 7 has been found in the cytogenetic laboratory at the RFHSM but trisomy 7 has been reported in at least 13 cases, in two cases as the only change (Mitelman, 1991).

Trisomy 9 in MDS has also been reported (Mitelman, 1991). The demonstration that neither of the two cells with 48 and 49 chromosomes in AML case 1 were clonal was of interest since trisomy 9, 22, 10 and 17 have all been reported in AML although the particular combination of trisomies seen in these cells has not been reported as clonal. High hyperdiploid clone investigated in case 6 was shown not to be clonal. High hyperdiploid clones are rare in MDS but have been reported (Mitelman, 1991).

Trisomy 4, found in a single cell in a remission marrow from a patient with ALL was also found to be a random event. In the cytogenetic database at the RFHSM among 291 remission samples from patients with ALL , with a normal karyotype, this sample was one of two with a single trisomic cell. In contrast 22 samples have had a single cell with structural change. The trisomy in remission in case 4 was not one of the trisomies found in the hyperdiploid clone at diagnosis and was not found in an earlier or subsequent remission marrow from the same patient, who remains in remission. Similar events have been described by Heerema et al and appear to be unimportant to prognosis (Heerema et al., 1993).

The total number of cells analysed cytogenetically in our cases was between 15 and 40. The detection of an abnormal cell in these samples suggested the presence of a clone of

between 15% and 6% (Hook, 1977). This supposition was not supported by interphase FISH in this investigation.

It can be concluded that single trisomies are most frequently due to random non-disjunctional events, while a minority may represent extremely minor clones in the leukaemic tissue sampled. The clinical significance of such cells is at present unclear.

Chapter 5

CLONAL INVOLVEMENT OF DIFFERENT CELL LINEAGES IN ACUTE LEUKAEMIA

5.1 Summary

The identification of the leukaemic progenitor cell may have important therapeutic implications. In this chapter FISH combined with morphology and/or immunology has been used to examine the clonal involvement of different cell lineages in acute leukaemia. Twelve patients with ALL and five with AML have been investigated. Four kinds of chromosomal abnormality were investigated, high hyperdiploidy in ALL (9 cases), the Philadelphia chromosome in ALL (3 cases), trisomy 8 in AML (3 cases) and translocations involving 11q23 in AML (2 cases). The presence of the cytogenetic abnormality in both myeloid and lymphoid lineages was demonstrated in two cases of Philadelphia chromosome positive (Ph+) ALL. In both cases the breakpoint occurred within the major breakpoint cluster region of the *BCR* gene (M-*BCR*). In the remaining case of Ph+ ALL with the minor breakpoint cluster region of the *BCR* gene (m-*BCR*) and in all cases of high hyperdiploid ALL, the abnormalities investigated were found to be restricted to the lymphoid lineage. In the 3 cases with AML and trisomy 8 and in two cases with AML and with the involvement of the *MLL* gene, the abnormalities investigated were found in myeloid lineage only.

5.2 Introduction

Acute lymphoblastic leukaemia (ALL) and acute myeloblastic leukaemia (AML) are haematological malignancies characterised by proliferation and accumulation of genetically altered, immature haematopoietic cells and their progenitors. ALL is an accumulation of primitive lymphoid cells and is presumed to arise in a cell committed to lymphoid development. In contrast AML is an accumulation of primitive myeloid cells and it is presumed to arise in a cell committed to the myeloid development. However, there also exist so called 'hybrid' or 'mixed' leukaemias showing features of both myeloid and lymphoid lineages which indicates that some acute leukaemias may arise in a pluripotent stem cell (Bradstock et al., 1981; Smith et al., 1983; Pui et al., 1984; European Group for the Immunological Characterization of Leukemias (EGIL) et al., 1995).

Only a proportion of acute leukaemias are chemocurable. It has been suggested that the chemosensitivity of different leukaemias is related to the proliferative properties of the cell in which neoplastic transformation has occurred (Jasmin, 1988; Greaves, 1993). Leukaemias originating in a pluripotent stem cell would be chemoresistant, while those originating from a committed progenitor cell would be relatively chemosensitive. The identification of the leukaemic progenitor cell can therefore, have important therapeutic implications.

Chromosomal abnormalities are frequently found in the malignant clone in haematological neoplasms. The Philadelphia chromosome results from the translocation t(9;22)(q34;q11). This abnormality accounts for 92% of chronic myeloid leukaemia (CML) cases. In ALL, the Philadelphia chromosome is detected in 3% -5% of childhood cases and 15% to 25% of adult cases and is associated with a bad prognosis (Secker-Walker et al., 1976; Bloomfield et al., 1977; Priest et al., 1980; IWCL3, 1981b; IWCL3, 1981a; Ribeiro et al., 1987; Secker-Walker, 1990). At a molecular level the Philadelphia chromosome is the product of the fusion of the part of the *ABL* gene located on chromosome 9 and the *BCR* gene located on chromosome 22. There are two different breakpoint cluster regions within the *BCR* gene: the 5.8 kb major breakpoint cluster (M-*BCR*) region and 35 kb minor cluster region (m-*BCR*).

Hyperdiploidy with more than 50 chromosomes identifies an important subgroup of patients with ALL. It is found in approximately 25% of childhood and in between 5%

to 11% of adult cases (Secker-Walker, 1994; Groupe Francais de Cytogenetique Hematologique, 1996; Secker-Walker et al., 1997). These patients have a better prognosis than that for any other ploidy group (Pui, Crist, 1992). The most common gains include chromosomes: X, 4, 6, 10, 17, 18 and 21.

Whole chromosome gain resulting in trisomy of one or more chromosomes occurs as clonal change at diagnosis in varying proportions of patients in all haematological malignancies (United Kingdom Cancer Cytogenetics Group, 1992). Established subgroups in AML include the following trisomies as the only change 4, 8, 9, 11, 13, 21 or 22 (Walker et al., 1994).

Abnormalities involving 11q23 are often observed in acute leukaemia. The t(6;11)(q27;q23) translocation has been chiefly found in AML M4 and AML M5 (Mitelman F, 1994). The gene involved in the fusion product is *MLL* while its partner gene is *AF6* located at the band 6q27 (Prasad et al., 1993). The t(11;17)(q23;21) is mostly found in AML M4 and AML M5 (Harrison et al., 1998).

Chromosome abnormalities can be detected using fluorescence *in situ* hybridization (FISH) in individual cells. FISH combined with morphology or immunophenotyping makes possible to detect chromosomal abnormalities in particular cell lineages (Kibbelaar et al., 1992; Price et al., 1992; van Lom et al., 1993; Nguyen et al., 1994). In this section FISH combined with morphology and immunphenotyping has been applied to investigate clonal involvement of different cell lineages in order to discover the probable level of commitment of the high hyperdiploid cases in ALL, in Philadelphia positive patients with ALL, in patients with trisomy 8 and AML and in patients with 11q23 abnormalities and AML.

5.3 Materials and methods

5.3.1 Patients

Patients diagnosed with ALL and high hyperdiploidy (9 cases), ALL and the Philadelphia chromosome (3 cases), AML with trisomy 8 (3 cases) and AML with 11q23 abnormalities (2 cases) were identified from laboratory records. Peripheral blood samples from three healthy individuals were used as controls.

5.3.2 Samples

Cells remaining from the cytogenetic investigation stored in Carnoy's fixative at -20°C were used for the metaphase FISH investigations. Investigations of lineage involvement were performed on cytospins which had been prepared from the mononuclear layer or high density fraction of cells from the patients' bone marrow. For the details of cell isolation, density gradient centrifugation, cell storage and cytospin preparation see section 2.2.1.

5.3.3 Investigation of the clonal involvement of different cell lineages.

In order to investigate the clonal involvement of different cell lineages, cells belonging to particular bone marrow cell lineages were first identified on cytospins by visualising either their morphology using May-Grunwald-Giemsa (MGG) stain or their immunophenotype using the alkaline-phosphatase anti-alkaline-phospatase (APAAP) technique. Subsequently, the same preparations were used for FISH investigation.

<u>MGG staining</u>

In order to visualise cell morphology, cytospins prepared from bone marrow aspirates were stained with MGG. For the details of the staining method see section 2.2.2. Following the MGG staining, cytospins were scanned and the localisation of cells of interest was recorded using low (x20) magnification. Their morphology was recorded using high (x100) magnification. The recording was achieved using a CCD camera coupled with a Zeiss microscope and an Apple Mac Computer equipped with the Smart Capture Software. When recording was complete, slides were destained in 100% methanol for 1 minute, air dried and prepared for FISH.

APAAP technique

Antibodies

To determine cell immunophenotype, monoclonal antibodies from DAKO were used as follows: T-lymphoid (CD3), B-lymphoid (CD19), myeloid (CD13) and erythroid (Glycophorin A).

Detection

For the details of the detection method see section 2.2.2. Following APAAP staining, the coverslips were removed by soaking in deionised water, air dried and prepared for FISH.

Fluorescence *in Situ* Hybridization (FISH)

Probes

Chromosome-specific alpha-satellite probes (Oncor, Gaithesburg) were obtained for chromosomes X, 6, 7 and 8. Chromosome X was targetted in cases 1-4 and 6-9 (see table 5.1), chromosome 6 in case 5 (see table 5.1), chromosome 7 in case 11 (see table 5.2) and chromosome 8 in cases 13-15 (see table 5.3).

Translocation probes (t(9;22)) identifying fusion of *ABL* with the minor (m) or the major (M) *BCR* were available from Oncor (Oncor, Gaithesburg). The probes were used in cases 10-12 (see table 5.2).

The YAC 13HH4 (Das et al., 1992; Kearney et al., 1992) containing *MLL* gene was used for the investigation of patients with 11q23 abnormalities (cases 16 and 17, table 5.4). Yeast cultures, a preparation of DNA and the labelling of the probe is described in section 2.2.3.

Hybridization and detection

Hybridization, detection are described elsewhere (section 2.2.3)

Control values

Control values for trisomic cells were calculated as described (section 2.2.3).

Scoring

At least 50 cells were scored in each lineage investigation. If less than 50 cells were available for the analysis of a given cell lineage, the analysis was regarded as a failure.

5.4 Results

5.4.1 Patients

Clinical data and karyotypes of patients with ALL and AML are shown in table 5.1-5.4. Karyotypes of patients investigated included, in ALL, high hyperdiploidy (9 cases) (table 5.1) and t(9;22) (3 cases) (table 5.2), in AML, trisomy 8 (3 cases) (table 5.3) and translocations involving 11q23 (2 cases) (table 5.4).

Table 5.1
Clinical data and karyotypes of patients with ALL and high hyperdiploidy investigated for lineage involvement.

case no	age(yrs) /sex	immuno phenotype	WBC x109/l	karyotype	stage	probe
1	3/F	Nk	12.3	53 XX,+X,+4,+6, del(6),+17,+21,+22 inc	diag	X
2	4/M	Nk	Nk	53,XY,+X,+14,+18,+21,+21,+mar1,+mar2	diag	X
3	4/M	Pre-B	7.8	63-66,XY,+X,der(1),+2,+4,+5,+6,+7,+8, +10,+11,+12,+16,+17,+18,+19.+20,+21,+22	rel	X
4	4/M	common	2.4	65,XY,+X,+1,+4,+5,+6,+6,+8,+9,+10,+10,+11, +12,+14,+add(17)(p),+18,+21,+21,+21,+22	rel	X
5	4/M	Nk	Nk	54,XY,+Y,+4,+6,+10,+14,+17,+21,+21	diag	6
6	5/F	Nk	7.9	52,XX,+X,+4,+18,+21 inc	diag	X
7	11/F	common	Nk	65-72,XX,+X,+X,+1,+1,+2,+5,+6,+6,+8, +9,del(9),+10,+11,+12,+16,- 17,+18,+19,+20,+20,+21,+21,+22,+2mar	diag	X
8	19/M	Nk	0.71	54-55,XY,+X,+4,+11,+21 inc	diag	X
9	23/M	Nk	Nk	79-87,XX,YY,-4,-8,-8,-11,-11,-12,-15,- 15,+22,+mar,+mar inc	diag	X

Table 5.2
Clinical data and karyotypes of patients with ALL and t(9;22) investigated for lineage involvement.

case no	age(yrs) /sex	immuno phenotype	WBC x109/l	karyotype	stage	probe
10	18/M	common	285	46,XY,t(9;22)	refract ory	M-*BCR-ABL*
11	38/F	common	Nk	44,XX, -7, t(9;22), -22	diag	7
11				54, XX,+X, t(1;7),+5,+8,+8, t(9;22), t(9;22), +11 del(12p), +12+17+19	follow-up	m-*BCR-ABL*
12	61/M	common	Nk	46,XY,t(9;22)	diag	M-*BCR-ABL*

Table 5.3
Clinical data and karyotypes of patients with AML and trisomy 8 investigated for lineage involvement.

case no	age(yrs) /sex	FAB	karyotype	stage	probe
13	34/M	M5	47,XY,+8	diag	8
14	49/F	M4	47,XX,+8, inv(16)	diag	8
15	69/M	M4	47,XY,+8, inv(16)	diag	8

Table 5.4
Clinical data and karyotypes of patients with AML and translocations involving 11q23 investigated for lineage involvement.

case no	age(yrs) /sex	FAB	karyotype	stage	probe
16	14/F	M4	46,XX, t(6;11)(q27;q23)	rel	YAC 13HH4
17	19/F	M4	46,XX, t(11;17)(q23;q21)	diag	YAC 13HH4

5.3.2 Controls

The control values of cells with detected pattern of hybridization signals for the probes used in the study are shown in table 5.5

Table 5.5
Control values of cells with the detected pattern of hybridization signals obtained from control blood samples from three females and three males.

probe	The hybridization pattern of signals in cells detected	cells with the hybridization pattern detected		
		Mean (M) (%)	Standard Deviation (SD) (%)	Control values(M+2x SD) (%)
alpha-satellite chromosome X (male controls)	2 signals	1.2	0.4	2
alpha-satellite chromosome X (female controls)	3 signals	1.1	0.4	1.9
alpha-satellite chromosome X (female controls)	4 signals	0.8	0.4	1.6
alpha-satellite chromosome 6	3 signals	0.9	0.3	1.5
alpha satellite chromosome 7	1 signal	3.2	0.3	3.8
alpha-satellite chromosome 8	3 signals	0.7	0.1	0.9
M-*BCR-ABL t(9;22) translocation probe*	M-BCR-ABL *fusion signal*	5.1	0.4	5.9
m-*BCR-ABL t(9;22) translocation probe*	m-BCR-ABL *fusion signal*	5.9	0.6	7.1
YAC 13HH4	YAC 13HH4 split signal	4.3	0.3	4.9

5.3.3 The results of APAAP/FISH and MGG/FISH investigation

Cases with ALL and high hyperdiploidy

Nine patients with high hyperdiploidy (cases 1-9) (table 5.1) were investigated with APAAP/FISH and MGG/FISH using an alpha-satellite chromosome specific probe to one of the chromosomes gained. The FISH investigation confirmed the presence of the relevant chromosomal gain on metaphase spreads in cases where such material was available (cases 3-4, 9-12). In the remaining cases the presence of the chromosomal gains was confirmed on interphase nuclei on cytospins.

The results of APAAP/FISH study are shown in fig 5.1. Significant values of cells with one additional signal to chromosome X (+X) (cases 1-4,6, and 8), two additional signals to chromosome X (+X+X) (case 7) or one extra signal to chromosome 6 (+6) (case 5) were observed in CD19+ cells only. In CD3+, CD13+ and Glycophorin A+ cells the numbers of cells with +X, +X+X or +6 were within control values. An example of APAAP/FISH investigation is shown in fig 5.2. Similar results were obtained with the MGG/FISH investigation (see fig 5.3). Significant values of cells with +X, +X+X, or +6 were observed in immature lymphoid cells only. In cells with myeloid or erythroid appearance, the numbers of cells with +X, +X+X or +6 were within control values. The example of MGG/FISH investigation is shown in fig 5.4.

Cases with ALL and t(9;22)

Three patients with t(9;22) (cases 10-12) (table 5.2) were investigated with MGG/FISH for the clonal involvement of cells with *BCR-ABL* fusion. The patients were first examined with the M-*BCR* and m-*BCR* t(9;22) translocation probes on metaphase spreads to confirm the translocation at the molecular level and to determine the *BCR* breakpoint. Two patients were found to have M-*BCR* breakpoint (cases 10 and 12) and one (case 11) with two t(9;22) and no normal 9 or 22 to have two copies of m-*BCR/ABL* fusion.

Subsequently the appropriate *BCR/ABL* probes were used on cytospins stained with MGG to determine the lineage involvement of cells with *BCR-ABL* fusion. The results of this investigation are shown in fig 5.5. In two patients with M-*BCR* (cases 10 and 12) elevated numbers of the cells with M-*BCR-ABL* fusion were observed in both lymphoid and myeloid cells (see fig 5.6). In the remaining patient with the m-*BCR* and

the two t(9;22) translocations significant values of cells with the double m-*BCR-ABL* fusion were observed in lymphoid cells only (see fig 5.6 and 5.7). The karyotype of this patient at diagnosis was 44,XY,-7, t(9;22) -22. The MGG/FISH analysis of this sample with alpha-satellite chromosome 7 specific probe revealed that -7 cells were present in cells of blast appearance only. No myeloid cells with monosomy 7 were found (see fig 5.8).

Cases with AML and trisomy 8

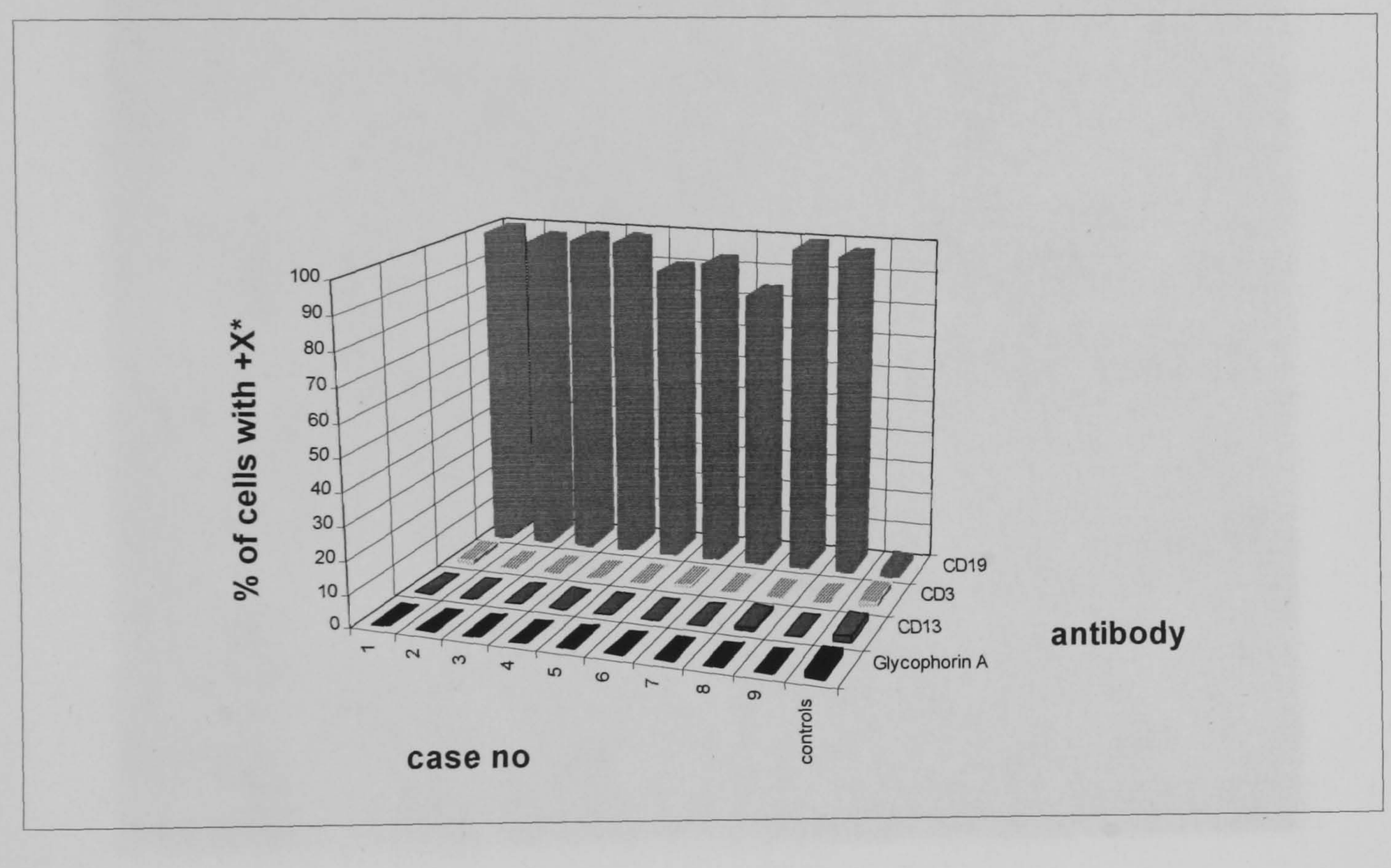
Three patients with trisomy 8 were examined with APAAP/FISH using alpha-satellite chromosome 8-specific probe for the clonal involvement of trisomic cells. The FISH investigation of metaphase spreads at diagnosis confirmed the presence of the gain of chromosome 8 in the great majority of metaphases in all cases. The results of the APAAP/FISH investigation are shown in fig 5.9. Significant values of cells with an additional signal to chromosome 8 (+8) were observed in CD13+ cells only. In CD3+, CD19+ and Glycophorin A+ cells the numbers of cells with +8 were within control values. The example of this investigation is shown in fig 5.10.

Cases with AML and translocations involving 11q23

Two patients with translocations involving 11q23, t(6,11)(q13;q23) (case 16) and t(11;17)(q23;q21) (case 17) were examined with MGG/FISH using YAC 13HH4 for the clonal involvement of the malignant cells. The location of the YAC 13HH4 was first determined by hybridization to peripheral blood from healthy controls. The YAC 13HH4 signal was confirmed to be on chromosome 11, at the q23 region (fig 5.11). The control values for cells with split YAC signal (identifying cells with the 11q23 region involvement) were obtained from mononuclear cells from peripheral blood of healthy controls (see table 5.5). The investigation of mononuclear bone marrow cells from two patients with 11q23 translocations revealed that significantly raised numbers of cells with the split YAC 13HH4 signal were observed in immature myeloid cells only (fig 5.12). The numbers of cells with a split signal in lymphoid cells were within control values. The example of this investigation is shown in fig 5.13

Fig 5.1

APAAP/FISH investigation of 9 cases of high hyperdiploid ALL. In 9/9 cases with high hyperdiploidy, cells with an additional signal to the alpha-satellite chromosome X specific probe were only seen in CD19+ve (B-lineage) cells. In CD3+ (T-lineage), CD13+ (myeloid) and Glycophorin A+ (erythroid) cells the numbers of cells with an additional signal to the alpha-satellite chromosome X specific probe were within control values.



* +6 (case 5), +X+X (case 7)

Fig 5.2

The results of APAAP/FISH investigation in case 7. Double gain of chromosome X is present in CD 19+ cells only. Cells positive with a given antibody are stained red. Green dots represent hybridization signals with alpha-satellite chromosome X specific probe. Note four dots present in CD19+ cells but only two present in CD3+, CD13+ and Glycophorin A+ cells.

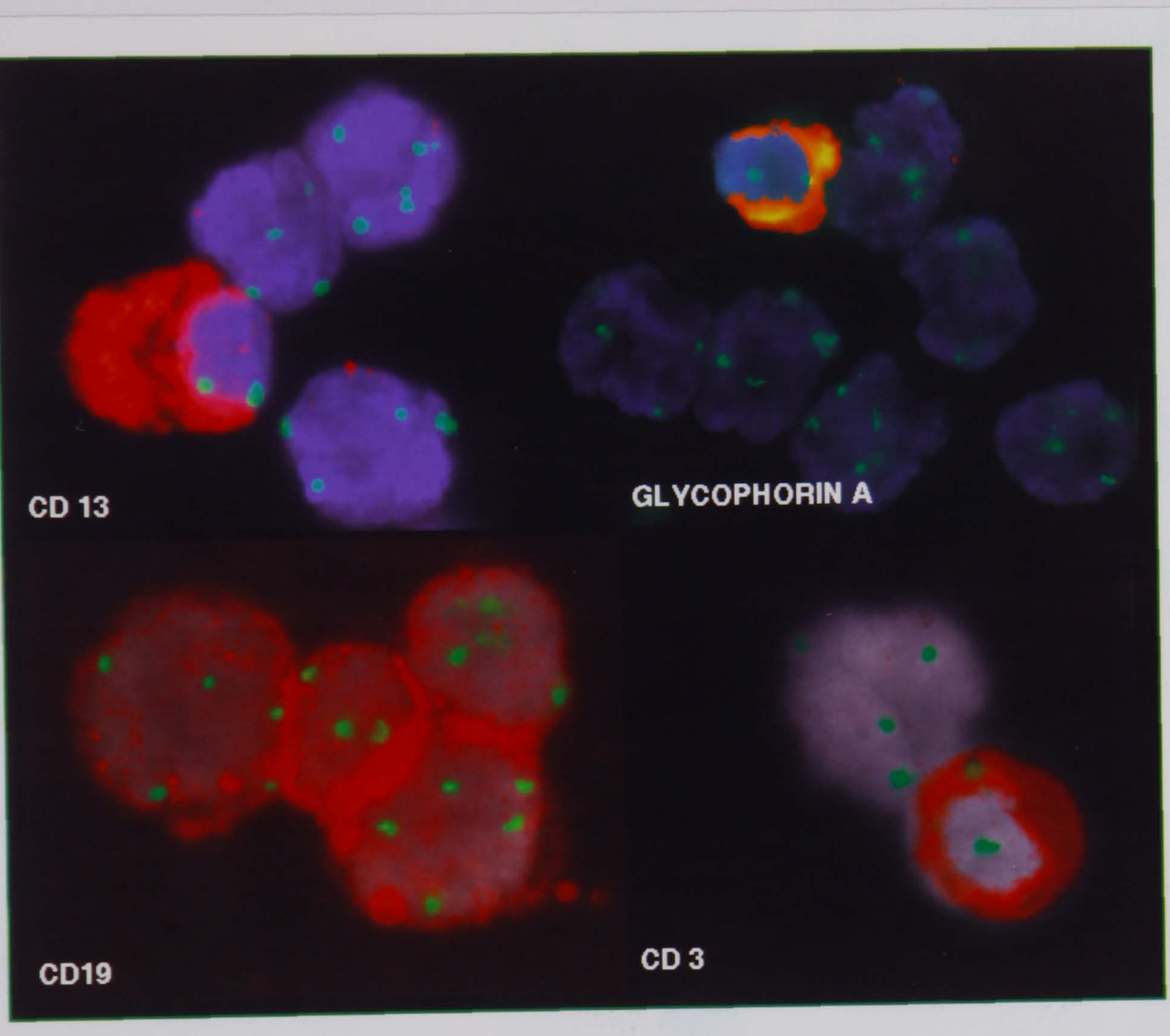
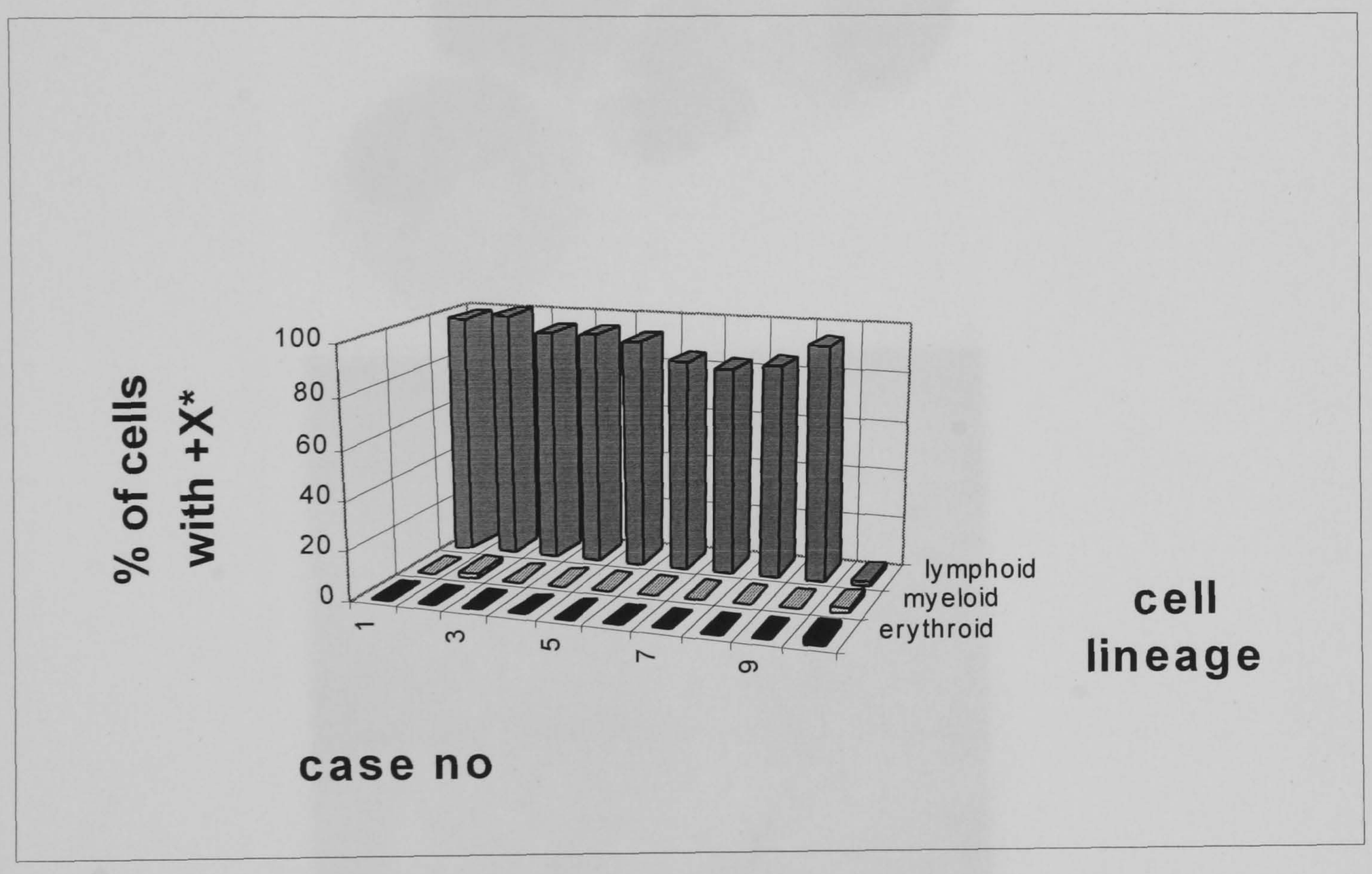


Fig 5.3

MGG/FISH investigation in ALL with high hyperdiploidy. in 9/9 cases cells with an additional signal to the alpha-satellite chromosome X* specific probe were only seen in lymphoid blasts. Cells with an additional signal to the alpha-satellite chromosome X specific probe were within control values in cells of myeloid or erythroid appearance.



* +6 (case 5), +X+X (case 7)

Fig 5.4

The results of MGG/FISH investigation in case 1. Gain of chromosome X present in immature lymphoid cells but absent in differentiating myeloid cells. Red dots represent the hybridization signals with alpha-satellite chromosome X specific probe.

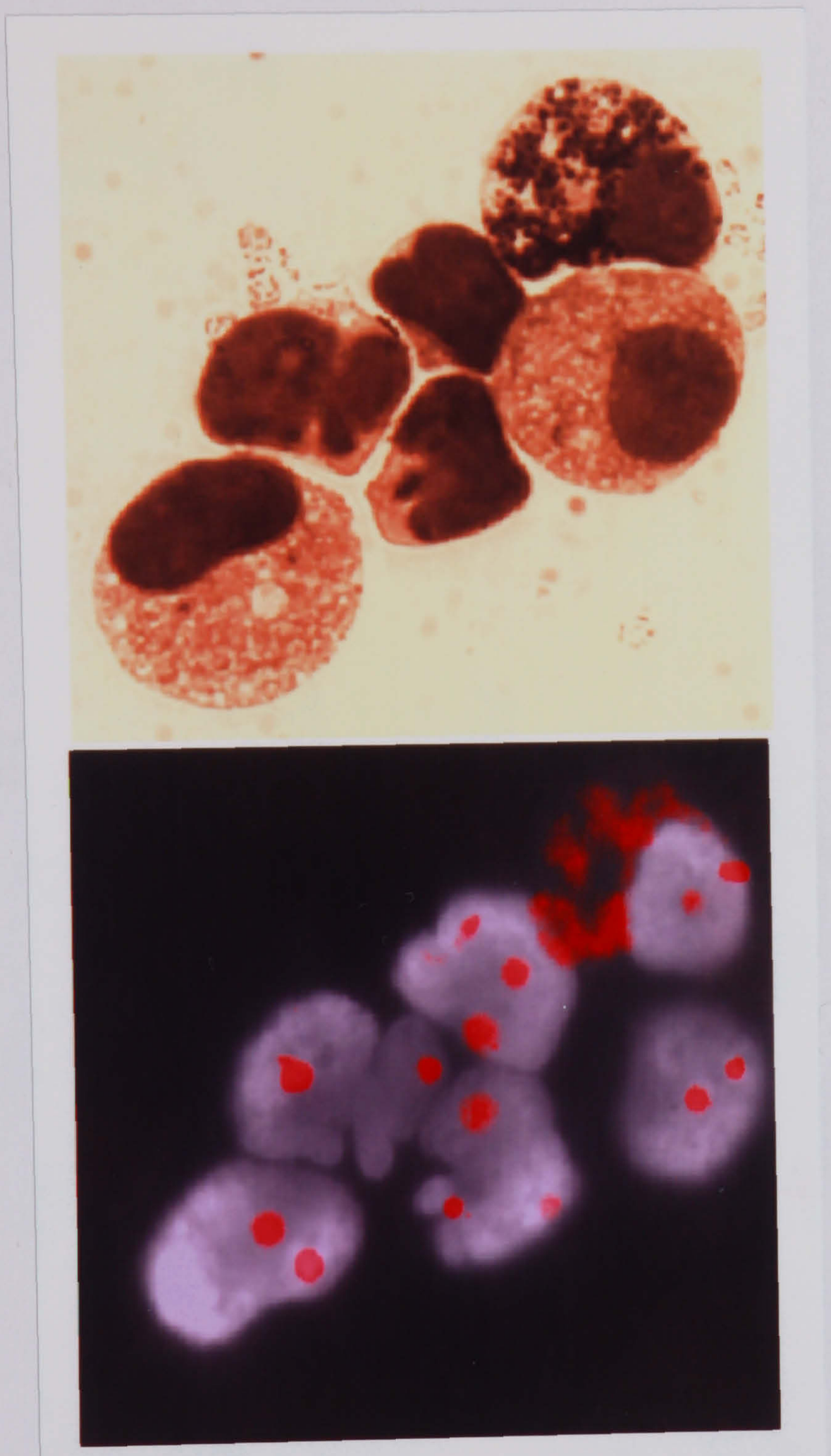


Fig 5.5

The MGG/FISH investigation of Ph+ ALL. Ph+ cases showed fusion of M-*BCR-ABL* in lymphoid as well as myeloid cells (cases 10 and 12) and fusion of m-*BCR-ABL* in lymphoid but not myeloid cells (case 11).

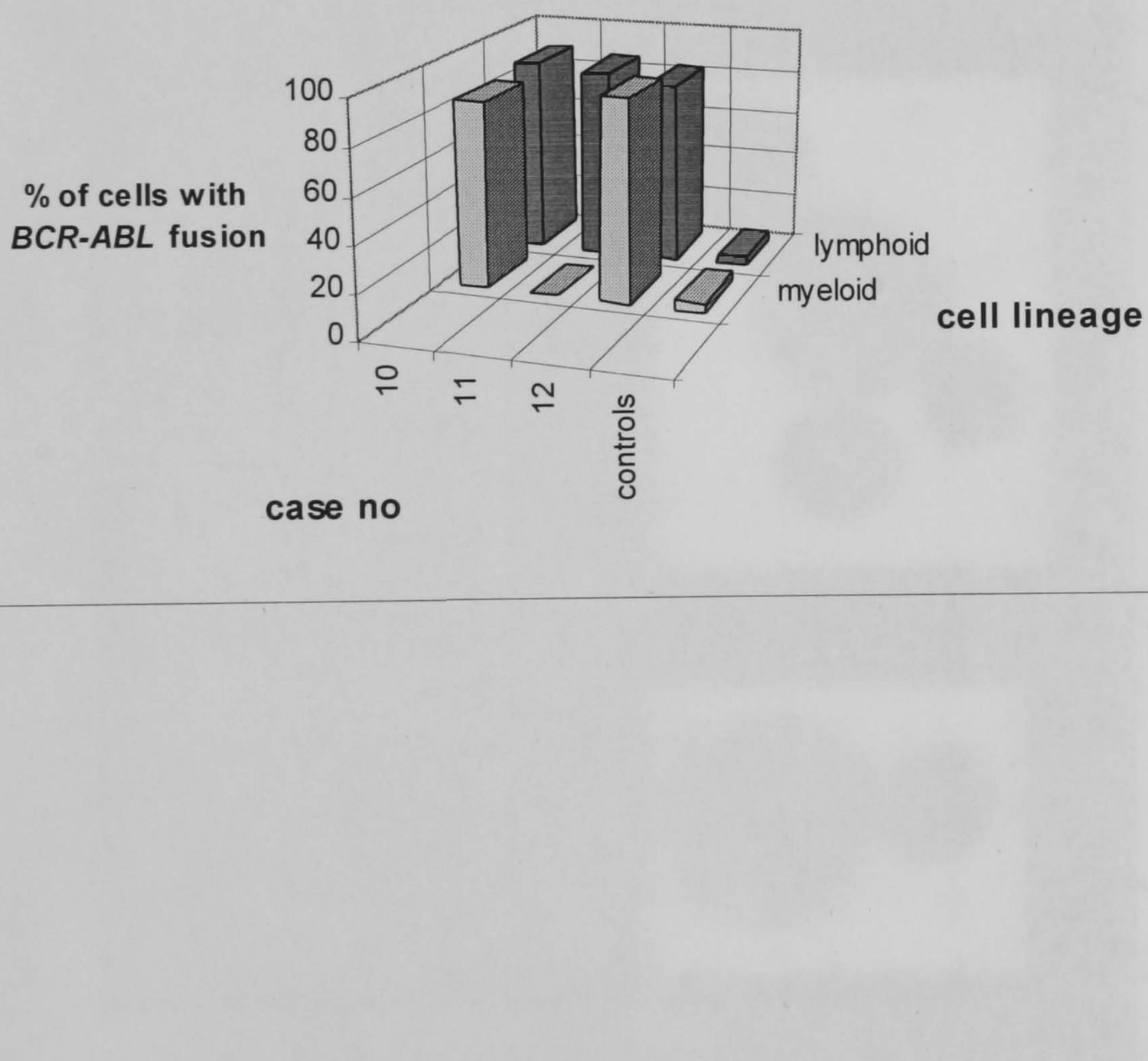


Fig 5.6

The results of MGG/FISH investigation in case 12. Translocation t(9;22) present in both in lymphoid and myeloid cells. Red dots represent hybridization signals with *BCR* and green with *ABL* probes respectively. Yellow signals represent *BCR-ABL* fusion.

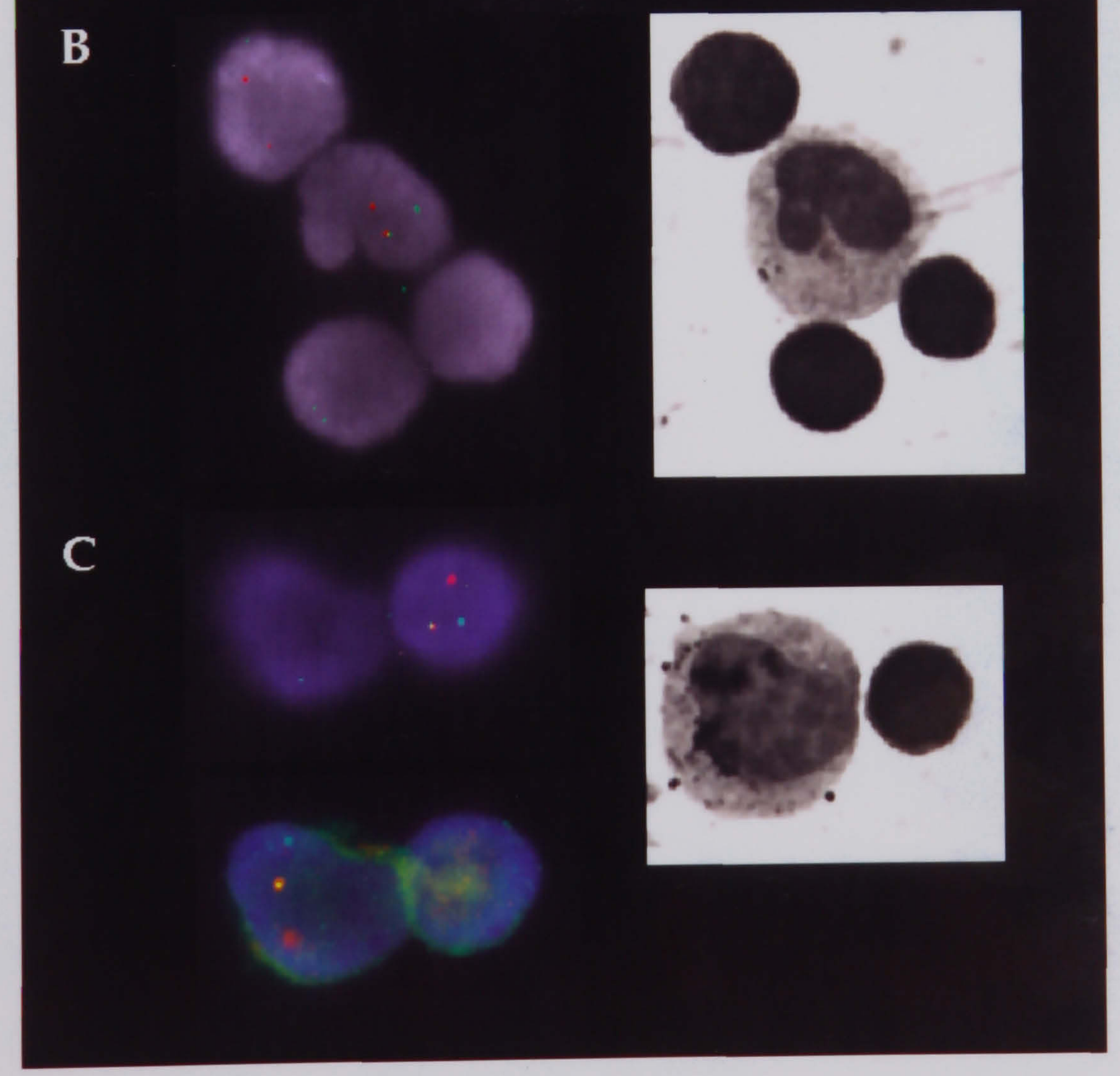


Fig 5.7

The results of MGG/FISH investigation in case 11. Double t(9;22) present in lymphoid blasts but absent in differentiating myeloid cells. Red dots represent hybridization signals with *BCR* and green with *ABL* probes respectively. Yellow signals represent *BCR-ABL* fusion.

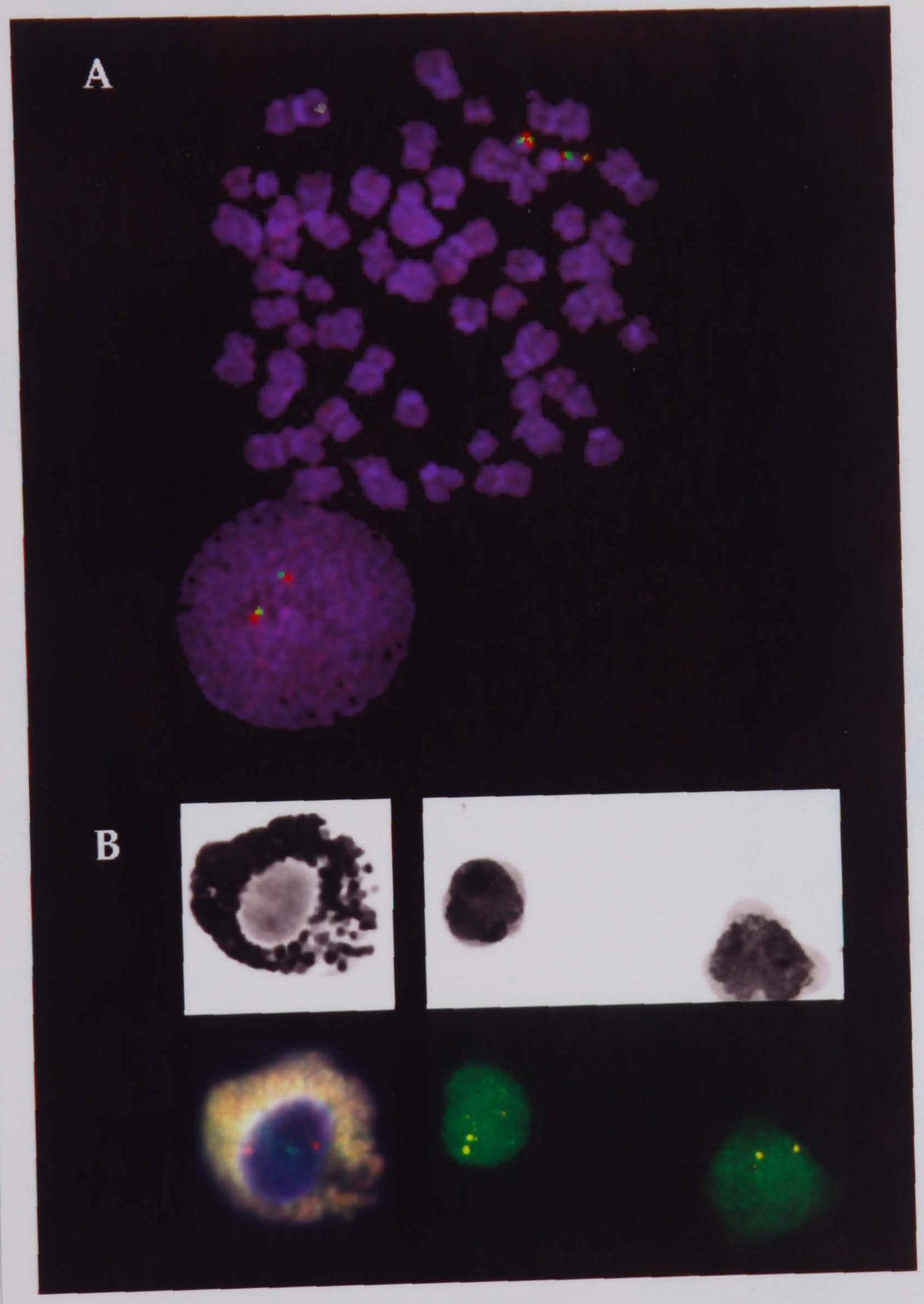


Fig 5.8

The results of the MGG/FISH investigation with alpha-satellite chromosome 7 specific probe in case 11. Red dots represent hybridization signals with alpha-satellite chromosome 7 specific probe. Note monosomy 7 present in blast cells only. Cells with myeloid morphology show 2 signals

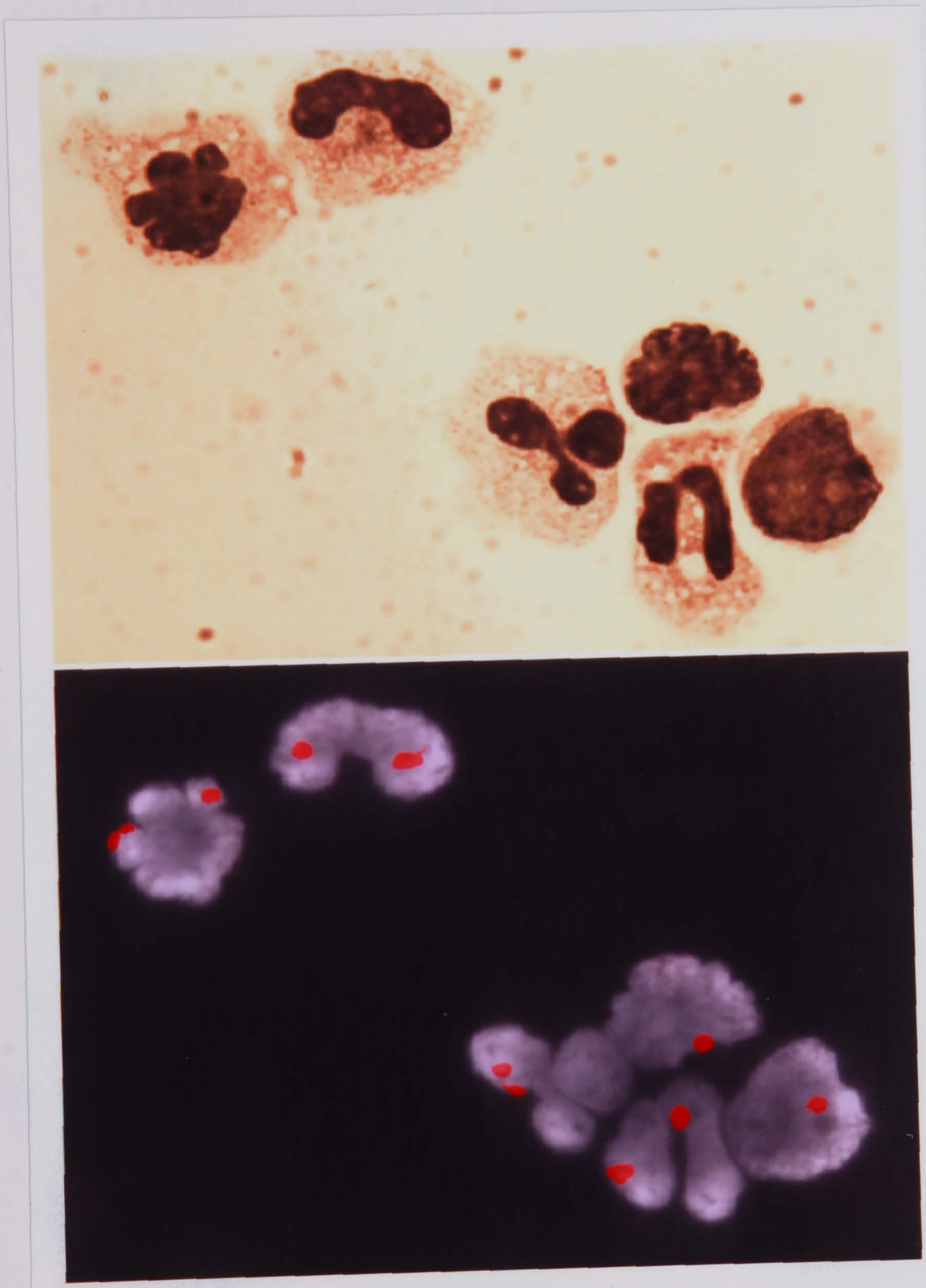


Fig 5.9

The APAAP/FISH investigation of cases with AML and trisomy 8 with alpha-satellite chromosome 8 specific probe. Significant values of cells with 3 signals were observed in CD13+ cells only. In CD3+, CD19+ and Glycophorin A+ cells, cells with 3 signals were within control values.

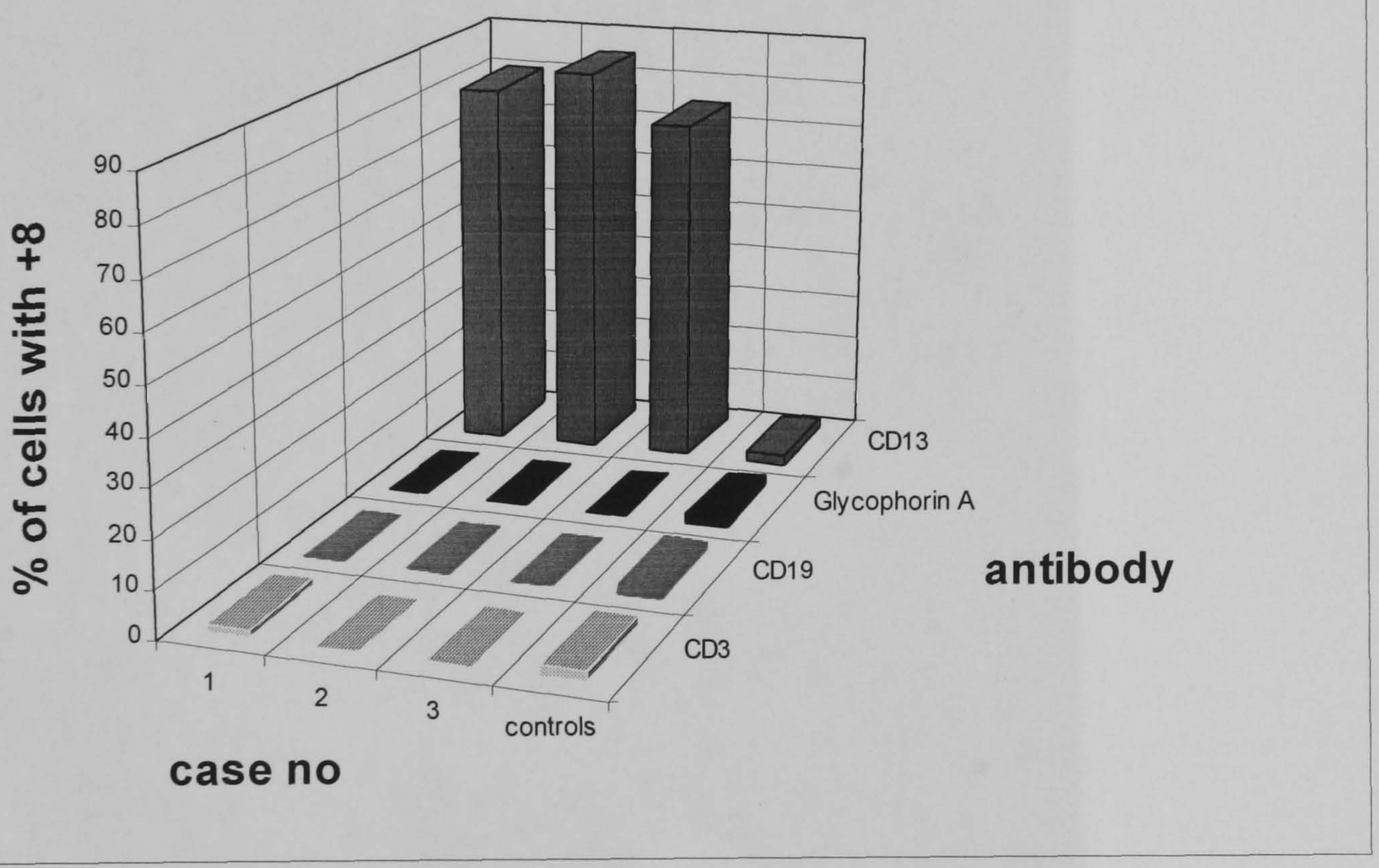


Fig 5.10

The example of APAAP/FISH investigation in case 13. Green dots represent hybridization signals with alpha-satellite chromosome 8 specific probe. Note that 3 signals are present in CD13+ cell only.

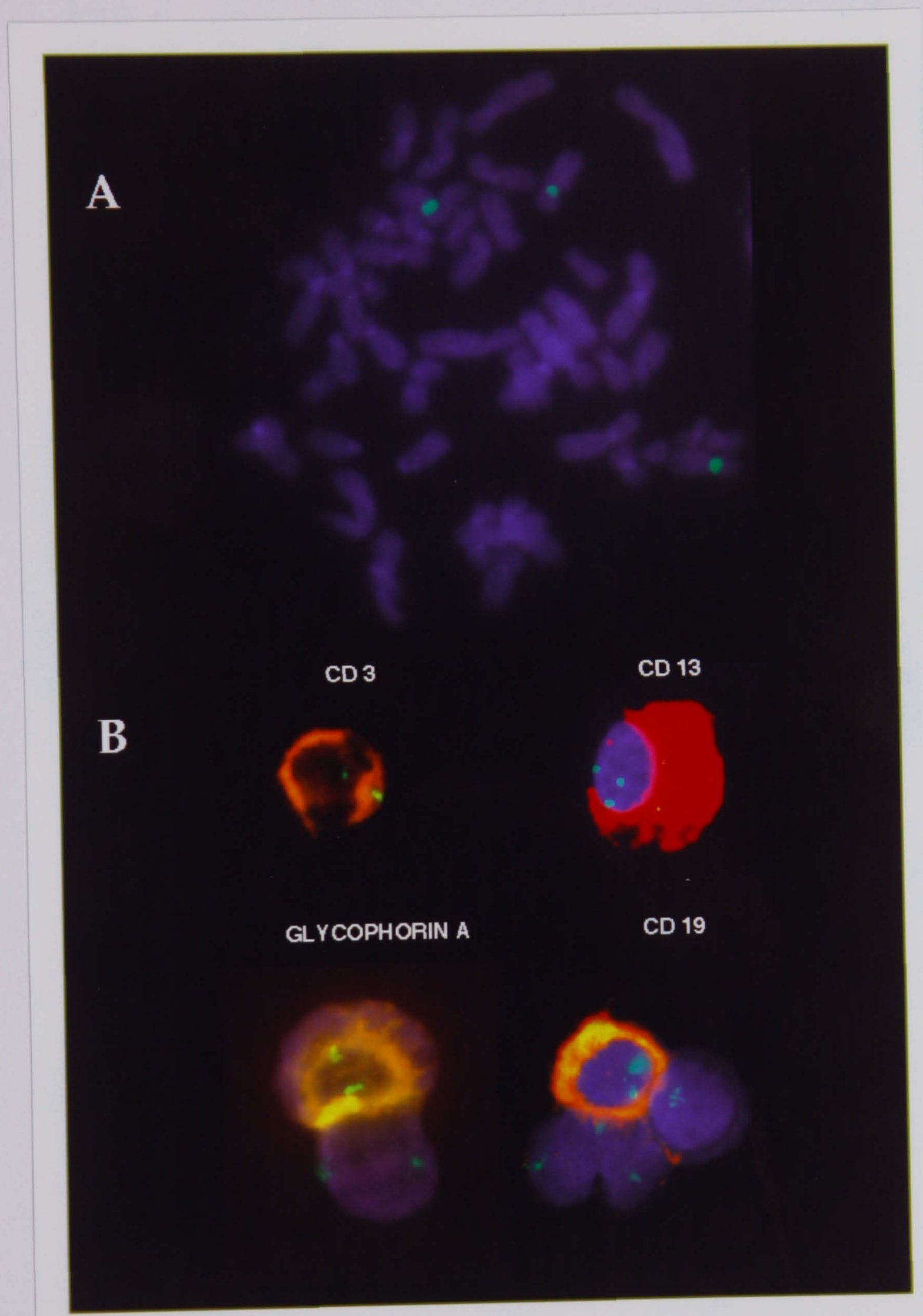


Fig 5.11

The localization of the YAC 13HH4

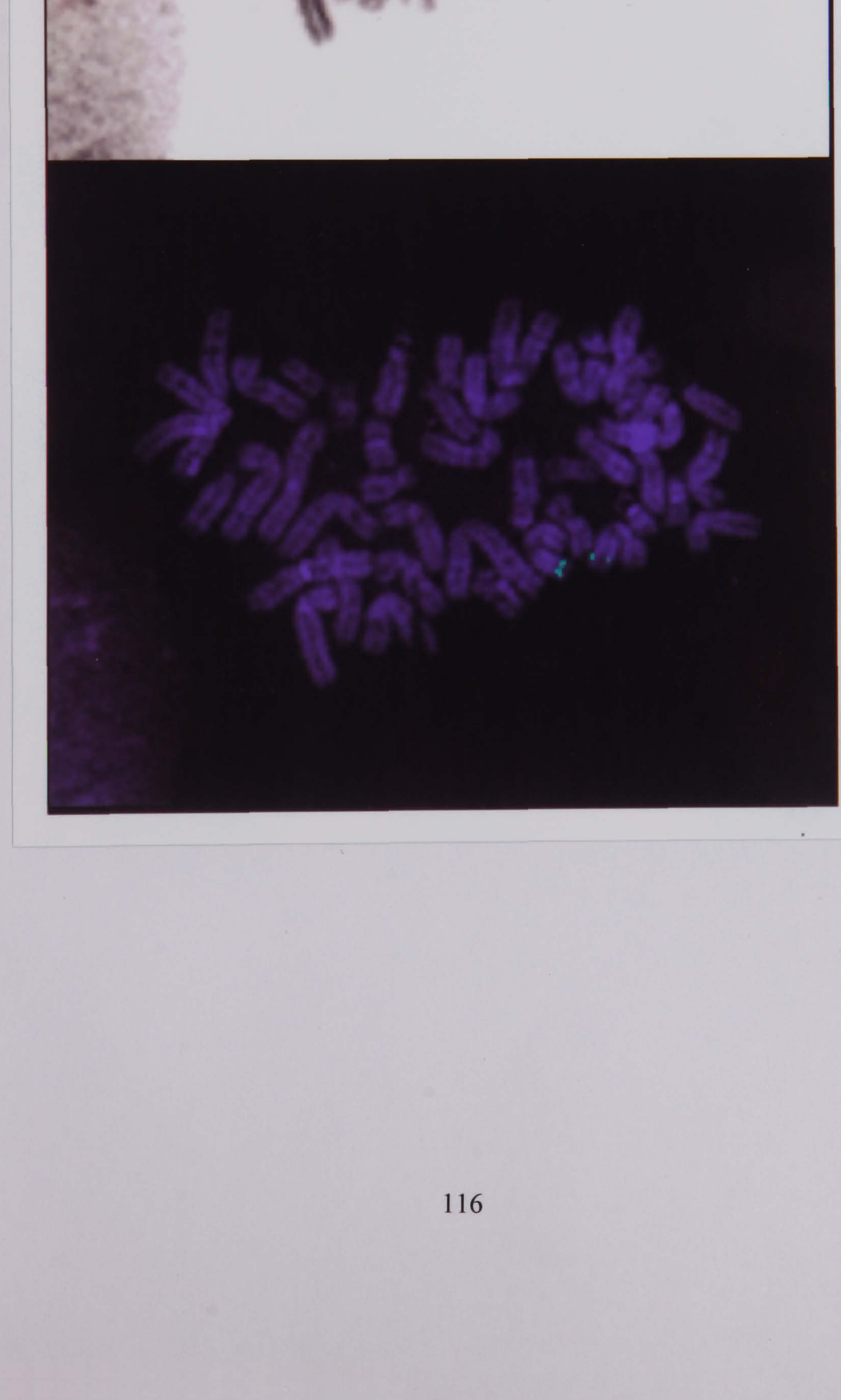


Fig 5.12

The results of MGG/FISH investigation of cases with AML and translocations involving 11q23. In both cases the significant values of cells with the YAC 13HH4 split signal was observed in cells of blastoid appearance. In lymphocytes, the numbers of cells with the split signal were within control values.

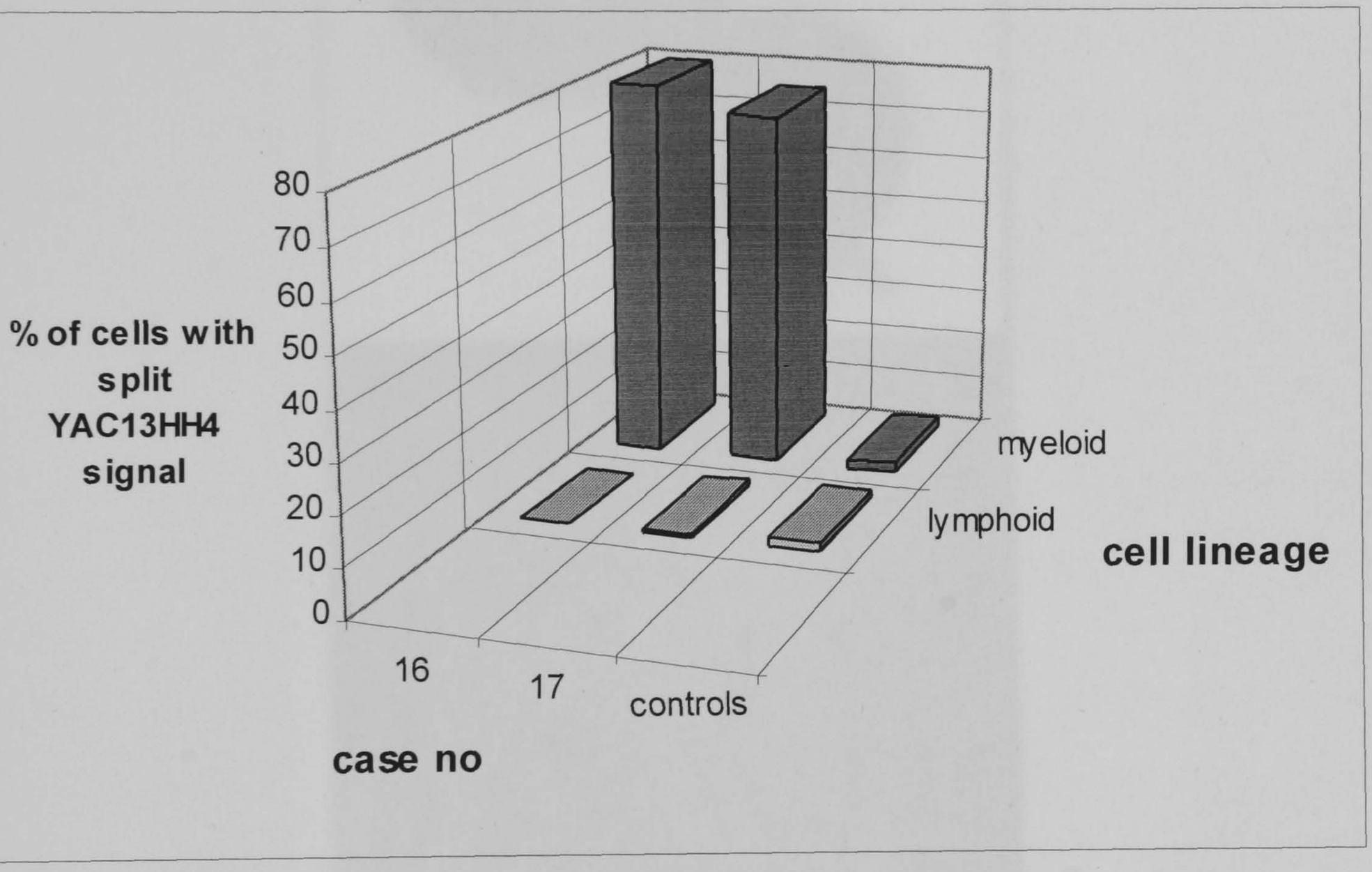
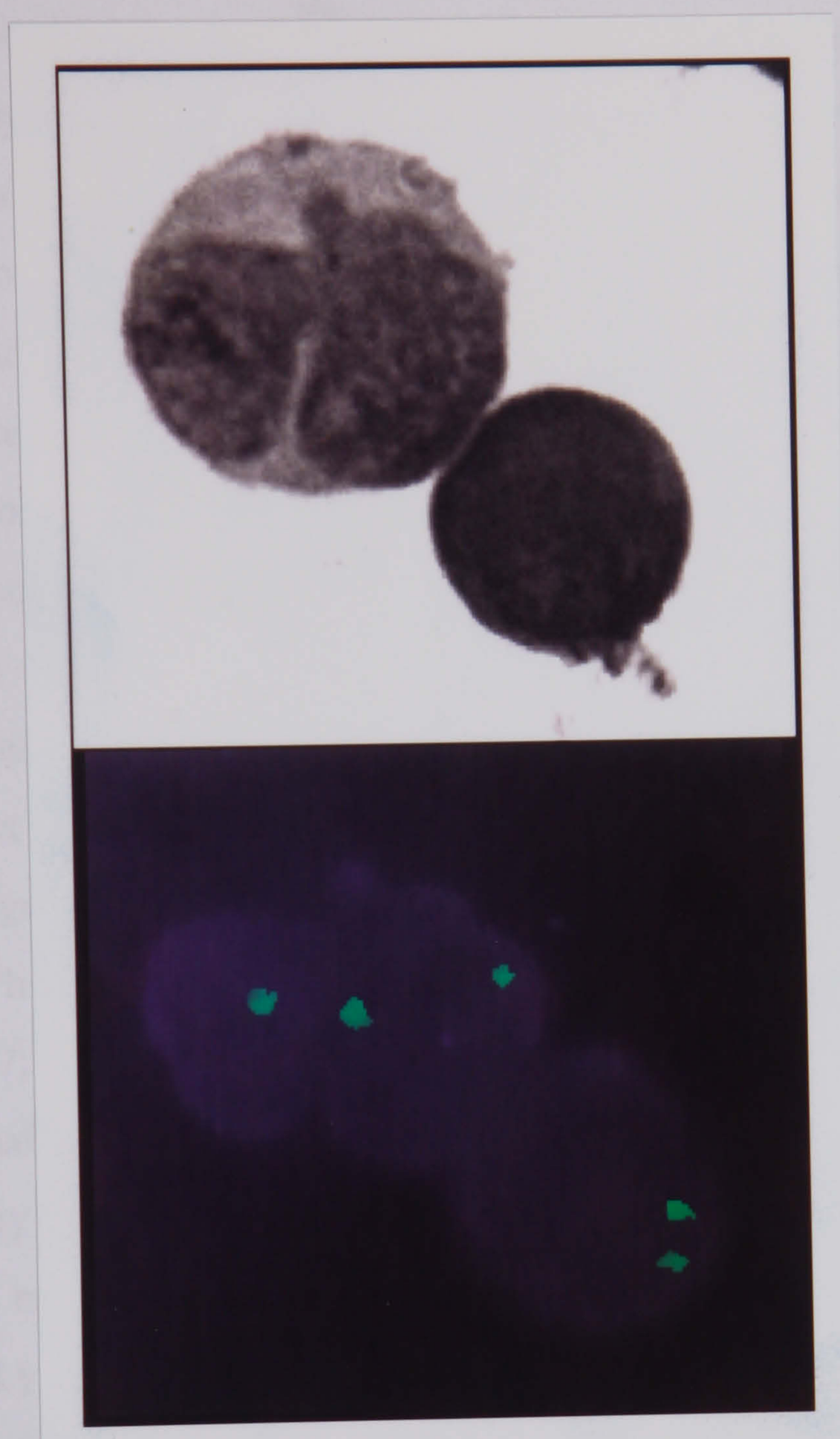


Fig 5.13

The results of MGG/FISH investigation using YAC 13HH4 in case 16



5.5 Discussion and conclusions

Twelve patients with ALL and five with AML have been investigated using FISH combined with immunphenotyping and/or FISH combined with morphology for the clonal involvement of different cell lineages. In all cases of AML the abnormality was restricted to the myeloid lineage. In the majority of cases with ALL the clonal abnormality was restricted to lymphoid lineage. The exceptions were found in Ph+ subgroup. In this group two cases showed involvement of both lymphoid and myeloid lineages. In both cases the breakpoint occurred within the major breakpoint cluster region of the *BCR* gene. The remaining Ph+ case with m-*BCR* was lymphoid only.

CML is a stem cell disorder, in which the target cell is capable of differentiating into myeloid, erythroid and lymphoid lineages. Studies with glucose-6-phosphate dehydrogenase isoenzymes have demonstrated that CML is a disorder of a pluripotent stem cell which is capable of differentiating into granulocytes, erythrocytes and macrophages (Fialkow et al., 1977). The involvement of B-lymphocytes has also been shown (Martin et al., 1980). The morphological picture of CML in lymphoid blast crisis is indistinguishable on morphological grounds from Ph+ ALL. It has been suggested that Ph+ positive ALL may be, in fact, CML presenting in a blast crisis (Chan et al., 1987; Clark et al., 1989).

In the present study myeloid and lymphoid lineage involvement was demonstrated by FISH/morphology in two cases of the Philadelphia chromosome positive ALL. In both cases the break had occurred within the major breakpoint cluster region of the *BCR* gene (M-*BCR*). Lymphoid restriction was shown in one case with the minor breakpoint cluster region of the *BCR* gene.

The lineage involvement of the Ph+ ALL has been the subject of the much debate. Cytogenetic studies of haemopoietic colonies derived from patients with Ph+ ALL have shown that Ph+ clones can be lymphoid restricted as well as occur both in lymphoid and myeloid compartment. Thus, Kalousek et al. (Kalousek et al., 1988) reported 4 cases with myeloid involvement and 4 without. Similarly, Dow et al. (Dow et al., 1989) reported 2 cases with myeloid involvement and 4 without. Three cases with lymphoid involvement only were reported by Kitano et al. (Kitano et al., 1988). In contrast, Tachibana et al. (Tachibana et al., 1987) reported two cases of Ph positive ALL and both of them with myeloid involvement. The investigation of the lineage

involvement of Ph+ ALL using molecular approach makes possible to distinguish between M-*BCR* (CML-like) and m-*BCR* (ALL-like) variant of Ph+ ALL. Three such studies were undertaken. In the first two, the cell compartments of Ph+ ALL were isolated by density gradient centrifugation and the presence of M-*BCR* or m-*BCR* rearrangements were detected by Southern blotting. Thus, Craig et al (Craig et al., 1990) reported two cases with M-*BCR* and m-*BCR* which were lymphoid restricted. Secker-Walker et al (Secker-Walker, Craig, 1993) reported two cases, one m-*BCR* and one M-*BCR*, both lymphoid restricted and two M-*BCR* cases with myeloid involvement. A different molecular approach was applied in the third study (Anastasi et al., 1996). The minor and major *BCR* were detected using FISH combined with morphology staining. The authors reported 4 cases of the M-*BCR* and 4 of the m-*BCR* Philadelphia positive ALL. In concordance with the study presented here, cases with m-*BCR* were lymphoid restricted and cases with M-*BCR* showed myeloid involvement. Secker-Walker et al. (Secker-Walker, Craig, 1993) reviewed the prognostic implications of lineage heterogeneity and the *BCR* breakpoint in Ph+ ALL and showed that there was no correlation between the breakpoint and survival. However, patients with myeloid involvement of clonal cells had significantly longer survival. This was later confirmed by Anastasi et al. (Anastasi et al., 1996). Paradoxically, it contradicts the theoretically predicted features of stem cell leukaemia (Jasmin, 1988; Greaves, 1993). These authors associated a lack of chemosensitivity with the involvement of the stem cell and relative chemosensitivity with the involvement of a committed progenitor cell.

Eight cases of hyperdiploid ALL investigated in this study were all shown to be lymphoid restricted. Lineage involvement of ALL has been the subject of three studies. Dow et al. (Dow et al., 1985) investigated types of glucose-6-phosphate dehydrogenase (G6PD) isoenzymes in 19 females heterozygous for this X chromosome-linked enzyme. Patients' karyotypes were hyperdiploid or pseudodiploid. The lymphoid blast cells from all cases displayed a single, (either A or B) G6PD isoenzyme type while in other cell lineages both A and B G6PD types were observed. Molto et al. (Martin-Henao et al., 1994) reported 2 hyperdiploid cases investigated with morphology/FISH technique using trisomy 7 and trisomy 8 as markers of clonality. The chromosomal abnormalities were found in lymphoid cells only. Knuutila et al

(Knuutila et al., 1993) investigated a male patient with ALL with t(5;14) and disomy X using the MAC technique. That technique, like the one used in the present study, makes it possible to study karyotypes in cells with an intact morphology. The chromosomal abnormalities were restricted to cells of lymphoid morphology only. Thus, the lymphoid restriction of clones in ALL other than Philadelphia-positive cases reported in this study is fully concordant with other studies published up to date.

All three cases of AML with trisomy 8 and two cases each with a different translocation involving 11q23 investigated in this study were found to be restricted to the myeloid lineage only. Lineage restriction in AML has been subjected to a number of studies, which differed in the techniques used. The MAC technique was used in three reports. Keinanen et al (Keinanen et al., 1988) investigated 12 cases with trisomy 8 and monosomy 7; Suciu et al. (Suciu et al., 1993) investigated 11 cases with various chromosomal abnormalities and Stamberg at al. (Stamberg et al., 1988) reported one case of AML with trisomy 8. In all three reports chromosomal abnormalities were found in cells of myeloid but not lymphoid lineage. FISH combined with morphology was utilised in two studies. Van Lom et al. (van Lom et al., 1993) investigated three cases with trisomy 8, monosomy 7 and -Y. Kwong et al. (Kwong, Chan, 1994) reported one case of AML with monosomy 7. In both studies the chromosomal abnormalities were found in the myeloid compartment but not in lymphoid cells. Interphase FISH combined with immunostaining was used in one study. Baurmann et al. (Baurmann et al., 1993) reported one case of AML with monosomy 7 restricted to the myeloid lineage. Chromosome X-linked polymorphism was used in five reports. Fialkow at al. investigated types of glucose-6-phosphate dehydrogenase (G6PD) isoenzymes in subsequent reports (Fialkow et al., 1979; Fialkow et al., 1981; Fialkow et al., 1987) in 1, 4, 27 cases of AML respectively. All reports were fully concordant showing a single G6PD type in myeloid blasts only. In two other reports using a similar approach, Kere et al (Kere et al., 1987) reported one case of AML with monosomy 7 and Najfeld et al. (Najfeld et al., 1988) reported one case of AML M7. Both cases were found to be myeloid restricted. Thus, the results of all above reports are concordant with the study presented here showing lack lymphoid involvement in AML

The data presented here, shows that a number of clonal chromosomal abnormalities in acute leukaemia are found only in one type of cell. This suggests that in these cases the acute leukaemia originates in a committed progenitor cell. Another possibility that cannot be ruled out is that the abnormalities might arise in a pluripotent stem cell which is then directed towards either the myeloid (in AML) or the lymphoid (in ALL) lineage only. Philadelphia positive ALL appears to be an exception to this rule. This study showed 2/3 cases in which the Philadelphia chromosome was found in both lymphoid and myeloid lineages, indicative of a pluripotent target cell.

In summary, lineage restriction appears to be the rule in the acute leukaemias but Ph+ ALL appears to be an exception.

Chapter 6

MINIMAL RESIDUAL DISEASE IN ACUTE LYMPHOBLASTIC LEUKAEMIA WITH HYPERDIPLOIDY

6.1 Summary

A theoretical analysis showed that false positive hybridization signals that limit the sensitivity of an interphase FISH assay represent probabilistically independent events. Therefore simultaneous targeting of two or more chromosome gains should result in increased sensitivity. The theoretical approach was tested by applying chromosome-specific probes singly, in pairs and as a triplet to three control samples. Control values (mean (M) + twice the standard deviation (SD) of positive cells in normal peripheral blood) were 1.6% to 2.0% in single probing (500 cells), 0.01% to 0.02% in double probing (10^4 cells) and 0% in triple probing (10^4 cells). Serial dilutions of bone marrow from a patient with a clone of >50 chromosomes in >85% of cells with peripheral blood were prepared. Each dilution mixture was probed with single, dual or triple probes and the clone was detected at dilutions of 10^{-1} (single, double and triple probing), 10^{-3} (double and triple probing) and 10^{-4} (triple probing). Subsequently, interphase FISH using double and triple probing was applied for MRD detection in patients with high hyperdiploidy. The chromosomes gained at diagnosis (13 cases) or in relapse (3 cases) were confirmed by FISH. Of 30 follow-up samples examined in remission 13 were positive for the hyperdiploid clone with between 0.01% and 0.06% of clonal cells. Positive samples in first remission were more frequent during the first 7 months of treatment (7/12) than thereafter (3/11).

6.2 Introduction

In this chapter the results of MRD investigation in patients with ALL and high hyperdiploidy using an adapted interphase FISH approach which was specific for the hyperdiploid clone are presented. By targetting three chromosomal gains simultaneously a sensitivity of 10^{-4} was achieved.

Acute lymphoblastic leukaemia (ALL) is a malignant disease of which 65% of children and 25% of adults achieve durable remissions and may ultimately be cured. (Chessells et al., 1995) Cytogenetic classification identifies a subgroup of patients with high hyperdiploid clones (>50 chromosomes) in which the malignant cells have at least 5 supernumerary (or trisomic) chromosomes. (Secker-Walker, 1994) Providing neither the Philadelphia translocation nor t(4;11)(q21;q23) is present these patients, whether children or adults, have a better prognosis than those with any other chromosomal finding. (Pui, Crist, 1992; Raimondi et al., 1996) An unusual feature of some high hyperdiploid clones is an apparent ability to remain dormant for many years. Relapse in this subgroup, which occurs in approximately 25% of children and 35% of adults, tends to be after the completion of treatment and even up to many years later (Dastugue et al., 1992). Also indicative of the relative inactivity of these clones is that second remissions, if they are achieved, tend to be longer than usual. (Secker-Walker, 1984) It was originally believed that the presence of any translocation would compromise the good prognosis of high hyperdiploid patients but more recent studies have not confirmed this. (Williams et al., 1986; Secker-Walker et al., 1989; Rubin et al., 1991) Therefore there is, at present, no way of identifying the patients in this subgroup who are destined to relapse.

It has been shown that failure to achieve clinical remission, 21 days from diagnosis, is associated with a poor prognosis in childhood ALL. (Legrand et al., 1994) It might therefore be expected that persistence of minimal residual disease (MRD), below the level of morphological detection, would be a similarly adverse indicator. Sensitive methods of detection of cytogenetic clones for MRD in ALL are at present limited to a minority of translocations, such as t(9;22)(q34;q11) and t(1;19)(q23;p13), which have been cloned and sequenced. These can be investigated by the reverse transcription

polymerase chain reaction (RT-PCR) with a sensitivity of 10^{-4} to 10^{-6} (Devaraj et al., 1995).

Additional copies of whole chromosomes, as found in high hyperdiploid clones, can be detected in interphase cells by fluorescence in situ hybridization (FISH) using chromosome-specific alpha-satellite probes. However, the detection of single additional chromosomes by interphase FISH cannot compete in sensitivity with RT-PCR for aberrant fusion products because of relatively high control values, caused by the presence of false positives (Saunders, Mauer, 1969; Heerema et al., 1993; Amiel et al., 1995; Zhao et al., 1995).

The aim of the study presented here was to develop a sensitive interphase FISH assay and to apply this technique for the detection of minimal residual disease in patients with ALL and hyperdiploidy.

The study involved the following steps. (i) The theoretical analysis of the sensitivity of an interphase FISH assay. (ii) Testing the sensitivity of a new approach to interphase FISH. (iii) The application of the above technique for MRD detection in patients with ALL and hyperdiploidy.

6.3 Analysis of sensitivity of interphase FISH

6.3.1 Background

Trisomies of certain chromosomes present in high hyperdiploid clones can be detected by interphase FISH using alpha-satellite chromosome-specific probes. Application of this technique allow the investigator to distinguish between normal and hyperdiploid cells (Rivera et al., 1991; Anastasi et al., 1992; Jenkins et al., 1992; Chen et al., 1993b; Escudier et al., 1993; Kibbelaar et al., 1993; Brizard et al., 1994). The distinction is based on the number of hybridization signals to a specific probe. Three signals identify a trisomy (as in a hyperdiploid cell), two signals identify a normal, diploid chromosome complement. The application of interphase FISH to normal controls results in two hybridization signals in a great majority of cells. However, cells with 0, 1, 3 or 4 signals are also found. The presence of cells with 3 signals ('false positives') limits the sensitivity of interphase FISH used for the detection of MRD in patients with hyperdiploid clones.

In this section an attempt to overcome the above limitation is presented. It is assumed that the acquisition of an extra hybridization signal ('false trisomy') by normal cells is a probabilistically independent event for each alpha-satellite chromosome specific probe applied. If two or more probes, targetting two or more chromosomes, are applied simultaneously, only cells showing trisomies of both chromosomes would be screened as 'positive'. It follows from the definition of probabilistic independence (see below, sections 6.3.2.2 and 6.3.2.3) that the simultaneous use of two or more probes should result in a decrease in number of cells with false trisomies. To test this assumption a model of hybridization signal distribution based on the conditional probability of independent events was introduced. Using this model the predicted frequencies of cells with extra hybridization signals using two chromosome specific probes simultaneously was calculated. The predicted frequencies were then compared with the frequencies of such cells obtained from normal controls.

6.3.2 Materials and methods

6.3.2.1 Frequencies of cells with one extra hybridization signal on normal controls

The frequencies of cells with an extra hybridization signal with a single probe were obtained for the X, 6 and 18 chromosome from peripheral blood samples from 3 healthy male controls from three peripheral blood samples (500 cells). Three blood samples were also used for the following probe pairs: X+6, X+18 and 6+18 (10^4 cells).

6.3.2.2 Conditional probability of independent events

Conditional probability of the simultaneous occurrence of two events if they are independent is defined as follows (Hayslett, 1997; Helms, 1997):

Events E_1 , E_2 , ...E_n are independent if

$$P(\bigcap_{i=1}^{n} E_i) = \prod_{i=1}^{n} P(E_i) \qquad (1)$$

where i,n εN, $P(\bigcap_{i=1}^{n} E_i)$ = is the probability of the joint occurrence of n events and

$P(\bigcap_{i=1}^{n} E_i) = P(E_1 \cap E_2 \cap ... \cap E_n)$,

$P(E_i)$ is the probability of the occurrence of an event E_i , and

$\Pi\ P(Ei) = P(E_1)\ x\ P(E_2) x ... x\ P(E_n)$.

Note that for n=2 formula 1 is expressed as:

$$P(E_1 \cap E_2) = P(E_1) x P(E_2) \qquad (2)$$

where E_1 and E_2 are events 1 and 2, $P(E_1 \cap E_2)$ is the probability of the joint occurrence of the events E_1 and E_2, $P(E_1)$ and $P(E_2)$ are the probabilities of each event occurring separately.

6.3.2.3 Probabilistic model of the distribution of hybridization signals

In order to apply conditional probability of independent events as described above for the description of the distribution of hybridization signals in an interphase FISH assay, the following definitions were introduced:

(i) A probabilistic event, E_i, is the gain of an extra hybridization signal by a given cell with a probe i.

(ii) The probability of a single event, $P(E_i)$, is the frequency of cells with a gain of one extra hybridization signal in a single probing.

(iii) The probability of a joint occurrence of two independent events, $P(E_1 \cap E_2)$, is the predicted frequency of cells with a gain of one extra hybridization signal with each probe in a double probing.

Thus, the predicted frequency of cells with a gain of one extra hybridization signal with each probe in a double probing is expressed using formula 2 as follows:

$P(E_1 \cap E_2) = P(E_1) \times P(E_2)$

where E_1 and E_2 are the gains of an extra hybridization signal by a given cell with probe 1 and 2, $P(E_1 \cap E_2)$ is the predicted frequency of cells with a gain of one extra hybridization signal with each probe in a double probing and $P(E_1)$ and $P(E_2)$ are the frequencies of cells with a gain of one extra hybridization signal in a single probing.

6.3.3 Results

The predicted (calculated using formula 2) and observed frequencies of cells with additional hybridization signals from each of the probe pairs are shown in table 6.1

Table 6.1

The observed and predicted frequencies of cells with one extra signal for the alpha-satellite chromosome-specific probe(s) (indicating trisomy for that chromosome) in three control samples

probe(s) specific to chromosomes	% of cells with one extra hybridization signal	
	observed	calculated*
X	1.2	NA
6	0.9	NA
18	1.0	NA
X+6	0.003	0.011
X+18	0.007	0.012
6+18	0	0.009

NA not applicable

* The frequencies of cells with extra hybrdization signal were calculated using formula 2 as follows:

For the probe pair X+6, $P(E_1)$ is the observed frequency of cells with 1 extra hybridization signal using probe X (1.2% see table 6.1), $P(E_2)$, the observed frequency of cells with 1 extra hybridization signals using probe 6 (0.9% see table 6.1). $P(E_1 \cap E_2)$ is the predicted frequency of cells with two extra hybridization signals (one from probe X and one from probe 6) (see formula 2). The formula 2 is then applied as follows:

$P(E_1 \cap E_2) = P(E_1) \times P(E_2) = 0.012 \times 0.009 = 0.00011 = 0.011\%$

The frequencies of cells with extra hybrdization signal for the other probe pairs i.e. X+18 and 6+18 were calculated in the same way.

The frequency of cells with an extra hybridization signal in a double probing was approximately 100 fold lower than the same frequency in a single probing for all the probe combinations tested (see table 6.1).

The probabilistic model tested gave a good prediction of the frequency of cells with an extra hybridization signal in a double probing. The predicted frequencies (range 0.009% -0.012%, mean 0.011%) were close to their observed counterparts (range 0% - 0.007%, mean 0.003%) .

6.4 Testing the sensitivity

6.4.1 Materials and methods

The new approach tested in this section involved applying two or three probes for the simultaneous detection of chromosomal gains, combined with a method for estimating the number of cells scanned. The accuracy of the estimation method and sensitivity of the assay were tested as described below.

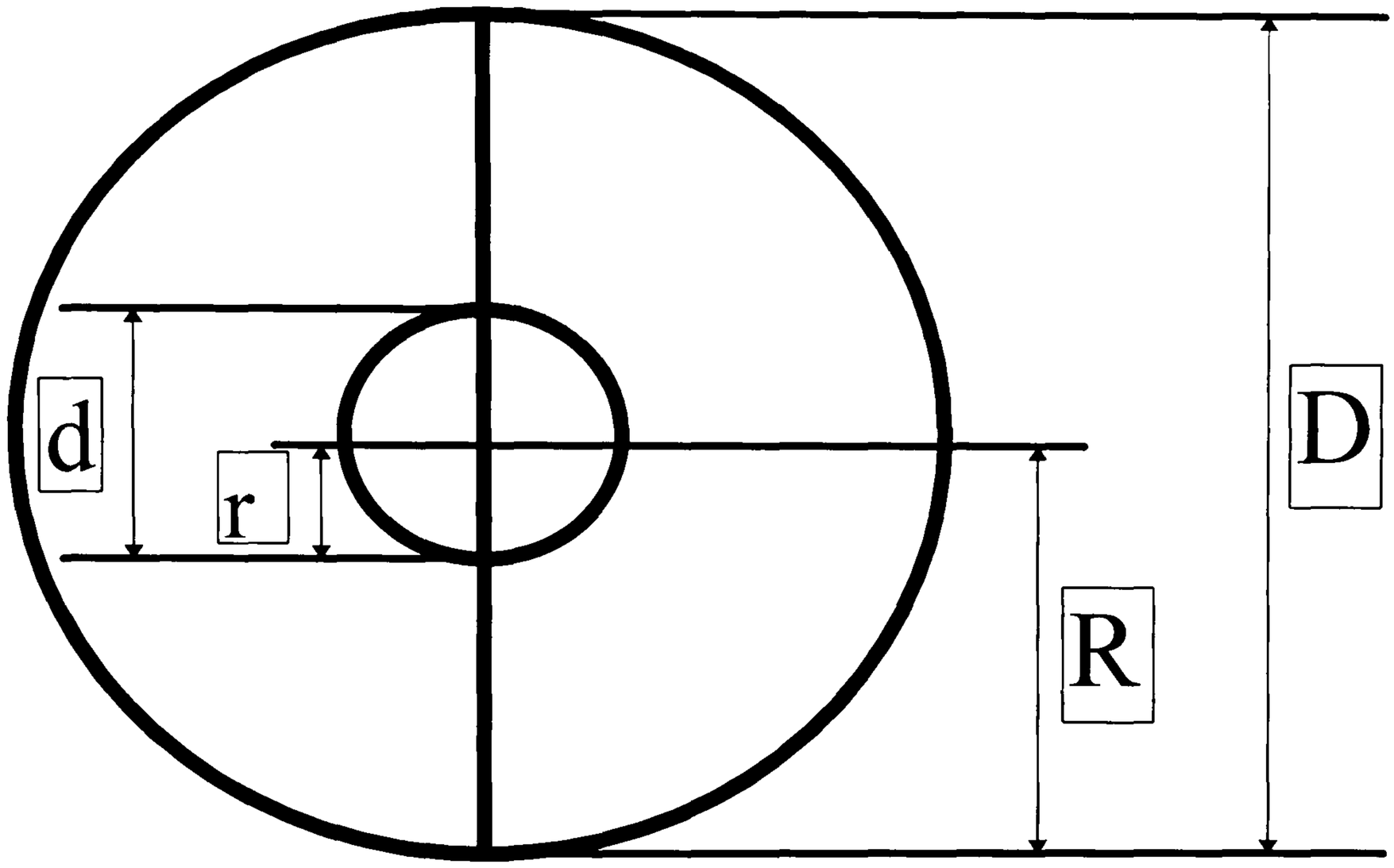
6.4.1.1 Cell number estimation

The method involved being able to score large numbers of cells. In order to avoid having to make repeated counts of the cells, the number of cells on a slide was extrapolated from representative fields of view. Cytospins were chosen because these give an even distribution of cells and the area of the cytospin is defined. It was assumed that the density of cells within a cytospin was constant and the number of cells per cytospin was calculated from the formula $N = n(D/d)^2$ (3) where N = the number of cells in a cytospin; n = the average number of cells from 10 fields of view using a x100 objective; D = the diameter of the cytospin and d = the diameter of the field of view. Formula 3 was obtained as follows. Figure 6.1 illustrates the geometrical relations between x100 objective field of view and a field of the cytospin. V_F-density of cells on a cytospin, V_f-density of cells in a x100 objective field of view, R-radius of a cytospin, r-radius of a x100 objective field of view, F-field of a cytospin, f-field of a x100 objective field of view. $N= V_FxF$ and $n= V_fxf$. Let $V_F=V_f =V=$ constant, then $N=(F/f)xn$. The cytospin and the x100 objective field of view are circles therefore $F=\Pi xR^2$ and $f=\Pi xr^2$. It follows that $N=(\Pi xR^2/\Pi xr^2)xn$ and $N=(R^2/r^2)xn$. Since $R=D/2$ and $r=d/2$ then $N=[(D^2/4)/(d^2/4)]xn$. Thus $N= (D^2/d^2)xn$, as was to be proved.

The accuracy of the method was tested for 20 cytospins by counting the number of cells present and comparing the actual number of cells with that estimated. An average cytospin contained 4000 cells requiring 3 cytospins to achieve a yield of 10^4 cells.

Fig 6.1

The method for approximating the number of cells scanned. Geometrical relations between x100 objective field of view and a field of the cytospin. (See section 6.4.1.1)



d- diameter of a x100 objective field of view
r- radius of a x100 objective field of view
D- diameter of a cytospin
R- radius of a cytospin

6.4.1.2 Sensitivity

In order to determine the sensitivity of the assay with regard to the number of probes used for the detection of chromosomal gains, the following dilution experiment was performed. Leukaemic marrow with the clonal karyotype 55,XY,+X,+6,+18,+21,inc was hybridized with probes to chromosome X singly, to chromosomes X and 6 as a pair and to chromosomes X, 6 and 18 as a triplet. Each hybridization yielded more than 85% cells with gains of one, two or three signals respectively. This marrow was mixed with peripheral blood mononuclear cells from one of the control samples to obtain the following dilutions (1:10; 1:100 1:1000 1:10,000). The following probes were hybridized and cells scored for each diluted sample: X singly (500 cells); X,6 paired and X,6,18 as a triplet (10^4 cells).

6.4.2 Results

6.4.2.1 Cell number estimation

Fig 6.2 illustrates the correlation between the number of cells scored and the number of cells estimated on 20 randomly chosen cytospins. The numbers of cells scored ranged from 859 to 7165 and the number estimated ranged from 880 to 8142. The correlation was good ($r = 0.9170$, $p<0.001$) confirming the reliability of estimating the number of cells in a cytospin from 10 representative fields of view.

6.4.2.2 Sensitivity

The results of the serial dilution tests using probes X singly, X,6 in pairs and X,6,18 as a triplet are shown in fig 6.3. The number of cells with one extra hybridization signal to each probe used was significantly above control values down to a dilution of 10^{-1} using a single probe and down to 10^{-3} using a pair of probes. Using the triplet, cells with three additional signals were detected in samples down to a dilution of 10^{-4}. Examples of FISH on metaphase chromosomes and on interphase cells from a patient with hyperdiploidy using probes singly, as a pair and as a triplet are shown in fig 6.4.

Fig 6.2
Correlation between the number of cells scored and the number of cells estimated on 20 randomly chosen cytospins ($r=0.9170$, $p<0.001$).

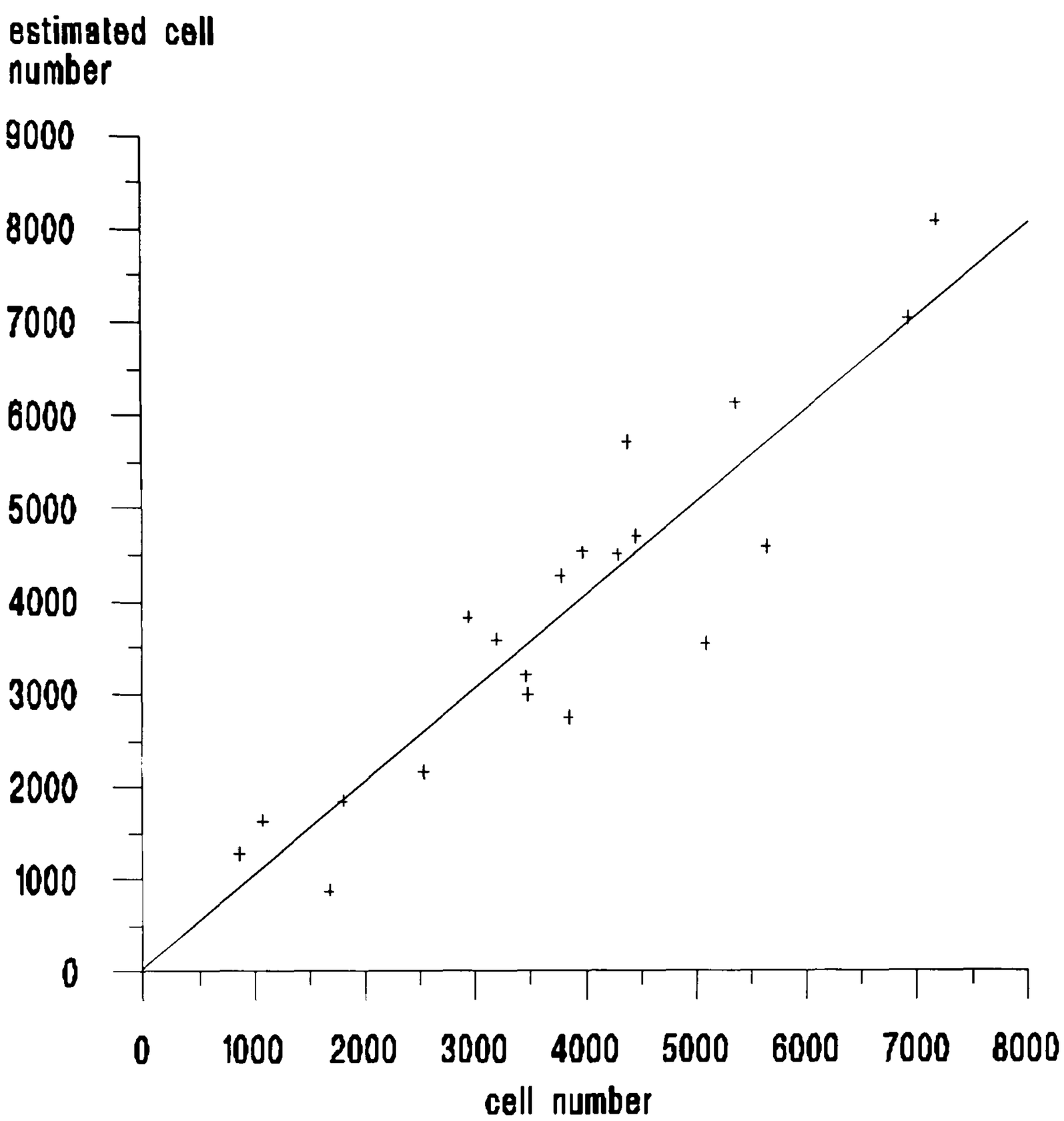


Fig 6.3
The results of FISH following serial dilutions of control cells with bone marrow with >85% hyperdiploid cells are shown for probes to chromosome X alone, X+6 together and X+6+18 as a triplet. The percentage of positive cells was significantly above control values using one, two and three probes at 10^{-1}, using two and three probes at 10^{-3} and using three probes down to 10^{-4} cells. Control values (mean + standard deviation x2) for the percentage of cells with gain of X, 6 and 18 hybridized to mononuclear cells of normal peripheral blood singly, in pairs and as a triplet are also shown

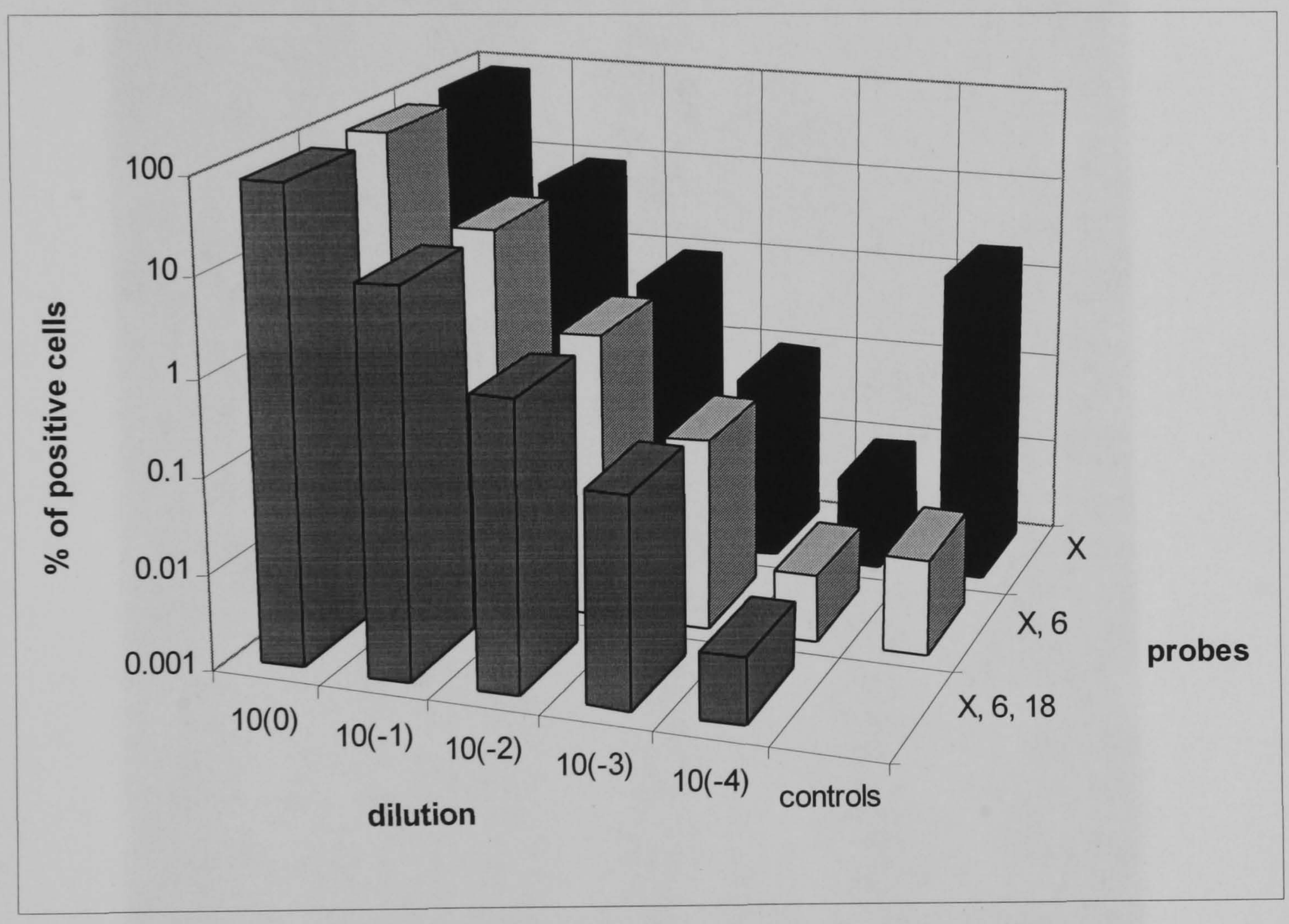
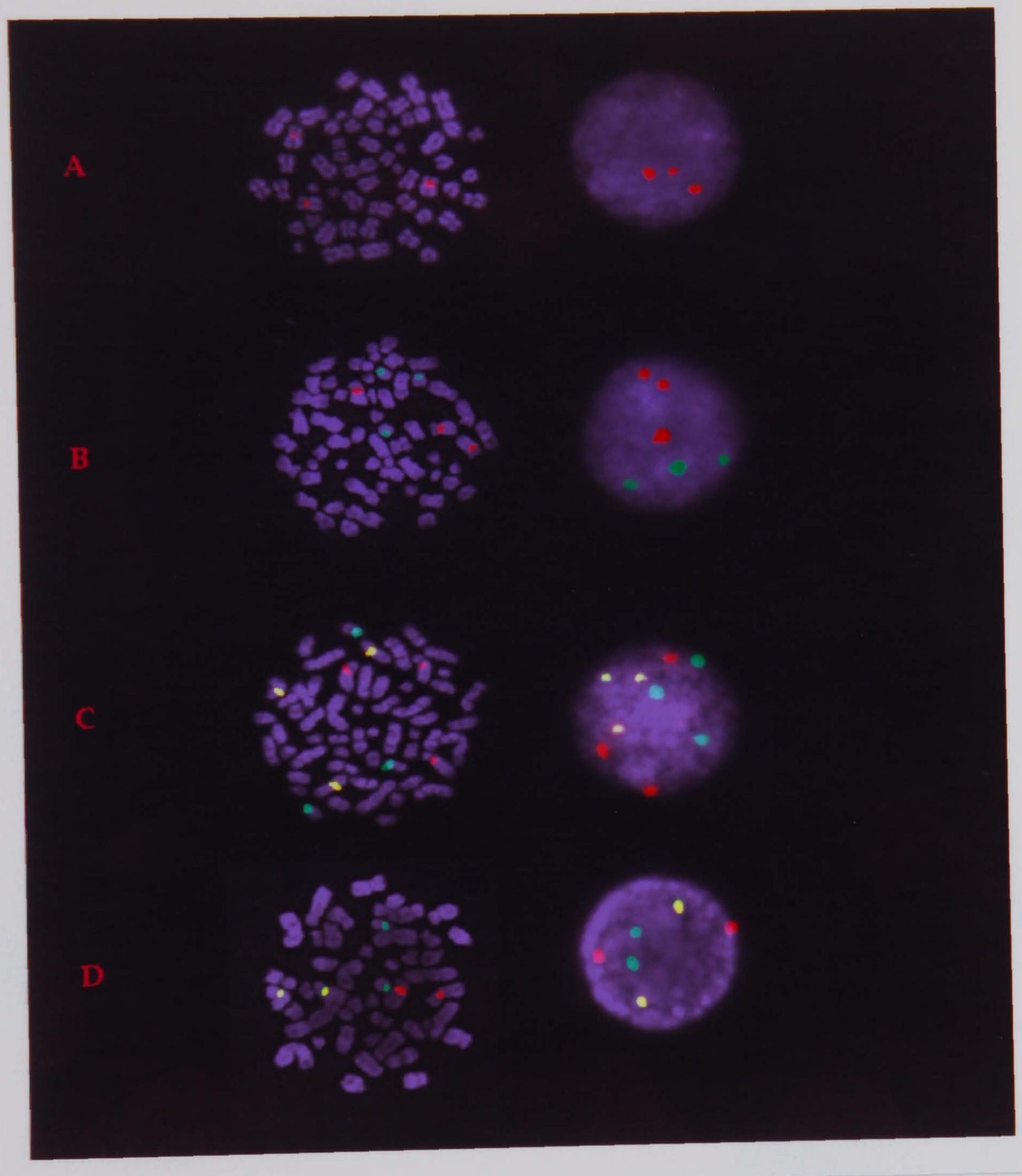


Fig 6.4
The results of FISH in case 6 (female) using chromosome specific centromeric probes at diagnosis on metaphase and interphase cells showing trisomy for chromosome 6 (red), 18(green), and X (yellow), A 6 hybridized alone, B 6+18 together, C X+6+18 together. D shows a normal female cell with two signals for each probe.



6.5 Application of the assay

6.5.1 Materials and methods

6.5.1.1 Patients

Bone marrow from patients with ALL found by metaphase cytogenetics to have a high hyperdiploid (>50 chromosomes) clone at diagnosis (13 patients) or in relapse (3 patients) were identified either in the cytogenetics laboratory at the RFHSM or by the Leukemia Research Fund, United Kingdom Cancer Cytogenetic Group (UKCCG) karyotype database in ALL. A total of 29 remission samples was obtained from these patients at periods ranging from 1 to 48 months after the initial sample. Control samples of peripheral blood were obtained from 3 healthy males and 3 healthy females

6.5.1.2 Control Values

Control values for the detection of trisomy X (or disomy X in males) with a single probe were obtained for the X chromosome from peripheral blood samples from 6 healthy controls (3 males and 3 females). Control values for each of the following autosomes 4, 6, 10, 17, 18 were obtained using three peripheral blood samples (500 cells scored from each sample). Three blood samples were also used for the following probe pairs: X,18 and X,6 and for each triplet: X,4,6; X,4,10; X,4,17; X,4,18; X,6,17; X,6,18; and X,17,18 (10^4 cells). Cells with three signals (or two for males using the X probe) for each probe used were recorded and expressed as a percentage of cells scored.

6.5.2 Results

6.5.2.1 Control Values

Control values for trisomic cells were calculated for each probe used, from the mean (M) and standard deviation (SD) of cells with one extra signal in three control samples. Control values were set at M+2SD. Using single probes, control values ranged from 1.5% to 2.1% as shown in table 6.2. Cells with simultaneous gain of two hybridization signals with probe pairs: X,6 and X,18 gave control values of 0.01% and 0.02% respectively. For each triplet (see section 6.5.1.2) no cell with all three additional signals was seen in 10^4 cells.

Table 6.2

Control values based on the mean and standard deviation of the number of cells showing an additional signal for each probe(s) used in the study

probe(s) specific to chromosomes	Mean (M) (%)	SD (%)	M+2xSD
X female	1.1	0.4	1.9
X male	1.2	0.4	2.0
4	1.6	0.2	2.0
6	0.9	0.3	1.5
10	1.1	0.3	1.7
17	1.2	0.2	1.6
18	1.0	0.2	1.4
X+6	0.003	0.005	0.013
X+18	0.007	0.005	0.017

Key
SD=standard deviation
M=mean

6.5.2.2 FISH in active disease.

The bone marrow used for FISH was part of the sample taken for karyotyping at diagnosis (13 cases) or at relapse (3 cases). Clinical data at diagnosis and karyotypes of the 16 patients studied is shown in table 6.3. The patients were 14 children (aged 1 year-10 years) and 2 adults (both aged 16 years), 11 were male and 5 were female. Immunophenotypes were common ALL (14 cases) or pre-B (2 cases). None of the patients had Down Syndrome. The chromosomal gains, detected cytogenetically and subsequently used for FISH in the MRD study, were confirmed using dual probes (cases 2 and 10) or triple probes (cases 1, 3-9, 11-16). The percentage of trisomic cells from diagnostic samples ranged from 75.4% to 91.4%, and from relapse samples from 31.9%-80.6%. (see fig 6.5). In one case (case 8) full cytogenetic analysis had not been achieved but FISH revealed a gain of chromosome X, which had not been identified cytogenetically.

6.5.2.3 FISH for follow up bone marrows

Part of the follow up bone marrow samples, taken for clinical assessment in remission, was received for FISH analysis. Thirty remission samples, one BM sample during CNS relapse and two in BM relapse were received. The results of FISH on follow-up samples are shown in fig 6.5.

The results were considered to be significant when the percentage of positive cells exceeded M+2SD of control values i. e. > 0% for triple probing > 0.01% for the X,6 pair and > 0.02% for the X,18 pair (see section 6.5.2.1).

For most samples a sensitivity of 10^{-4} was achieved following triplet probing (see section 6.4.2.2). Exceptions were samples from cases 2 and 10 to which only paired probes were applied (sensitivity of 10^{-3}). During remission, at these levels of sensitivity, a small percentage of hyperdiploid cells was detected in at least one sample in 8 out of 16 patients tested. The percentage of trisomic cells detected ranged from 0.01% to 0.06%. Positive samples in first remission were more frequent during the first 7 months from diagnosis (7/12 positive) than later on (10-24 months from diagnosis 3/11 positive). Only one of the five samples taken at the end of treatment or later showed cells with additional signals to the probes used. This patient, case 11, had 0.06% clonal cells at the end of treatment. It was of interest that this patient relapsed 6 months later. Serial samples (available in 8 cases) showed a tendency for the clone to diminish or disappear with time. Exceptions were case 11, who subsequently relapsed as described above and case 6, in whom 0.01% of clonal cells were detected 18 months from diagnosis following previous serial negative samples. This patient remained well 30 months later. In 8 patients the clone was apparently eliminated by chemotherapy and in another by bone marrow transplantation.

Examples of a field of interphase cells at diagnosis and in remission, probed with the triplet X,17,18 are compared in fig 6.6. There was no difference in age, sex, or initial WBC's (> or < $10x10^9$/l) between patients with positive and negative samples.

6.5.2.4 FISH in subsequent relapse

Three patients were investigated in relapse. Case 2 in CNS relapse, showed no abnormal cells in the bone marrow either by morphology or by FISH. Case 15, initially investigated in first relapse, had a second BM relapse 28 months later. This was

chromosomally normal but FISH showed it to have the trisomies identified previously in 10% of cells. Case 11, in BM relapse 30 months from diagnosis was found to have the gains described at diagnosis in 3/50 metaphases investigated by cytogenetics and FISH revealed 14% of clonal cells. Interestingly enough, this patient had a positive remission sample as the only one out of 3 patients investigated at the end of treatment.

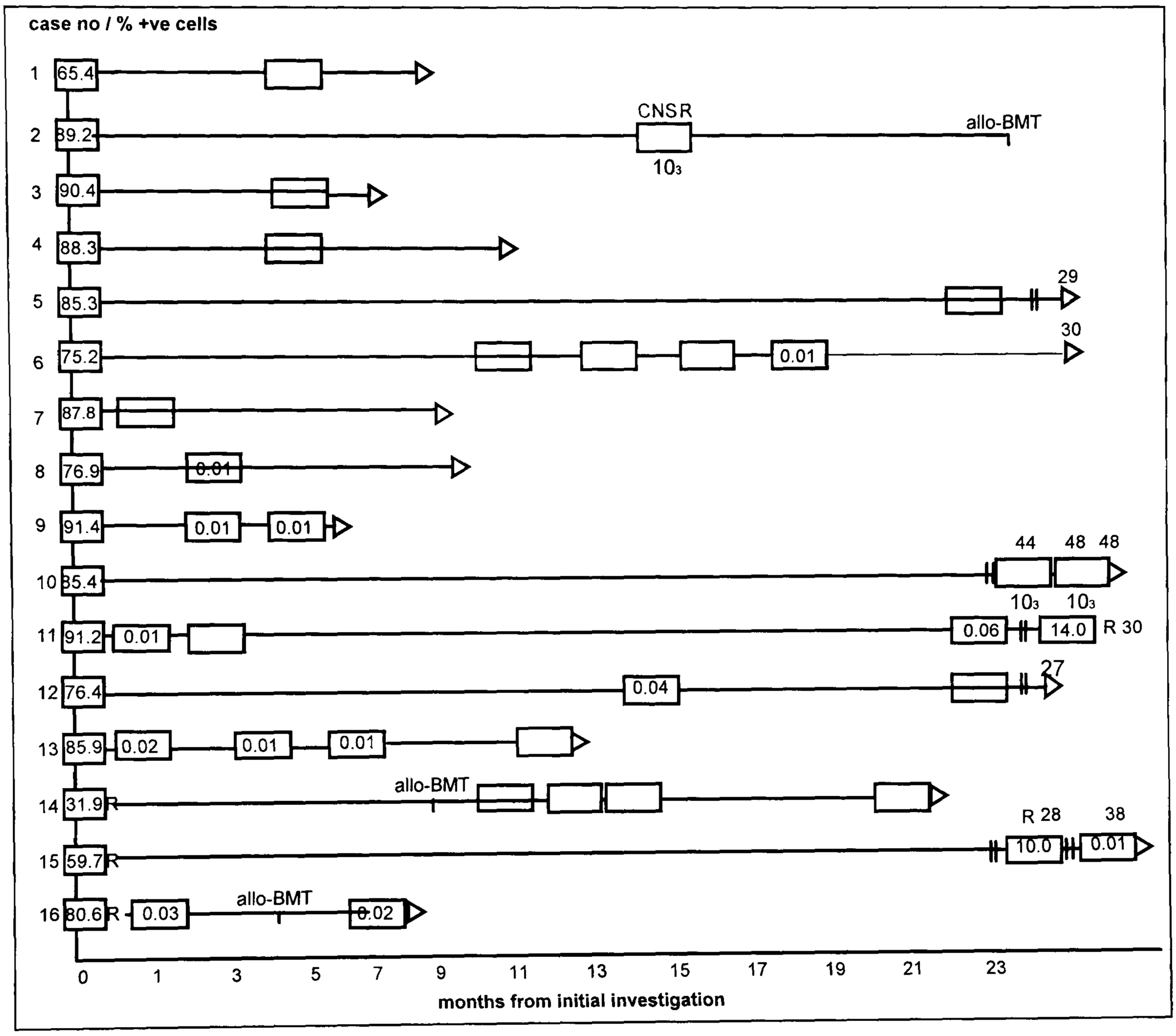
Table 6.3
Clinical data and karyotypes showing probes used for FISH.

case no	age (yrs) /sex	WBC $x10^9/l$	immuno	karyotype	probes specific to chromo somes
1	1/M	9.0	common	51-54,XY,+X,+4,+6,+14,+21,+22,+mar	X,4,6
2	1/M	55.8	common	56-59,XY,+X,+4,+10,+12,+16,+18,+21,+21, +mars	X,18
3	2/M	44.3	common	53,XY,+X+6,+10,+14,+17,+21,+21,+21	X,6,17
4	3/F	7.4	common	55,XX,+X,+6,+14,+17,+18,+21,+22,+mar1	X,6,18
5	3/M	3.6	common	54,XY,+X,+Y+6,+del(10)(q22q26),+14,+17, +21,+21	X,6,17
6	3/F	42.8	common	56,XX,+X,+4,+6,+8,+10,+14,+17,+18,+21, +21	X,6,18
7	3/F	23.8	common	57,XX,dic dup(1)(q10q42),+4,+6,+8,+9,+10, +14,+17, +18,+20,+21,+21	4,6,17
8	4/M	9.8	common	57-59,XY,+4,+5,del(6)(q13q15),+8,+9,+14, +15,+17, +18,+21,+21 inc	X,4,18
9	4/M	5.7	common	52,XY,+X,+6,+14,+17,del(17)(q25),+18,+21	X,6,18
10	8/M	6.7	common	51 XY,+X,+6,+14,+21,+21	X,6
11	10/M	9.5	common	54,XY,+X,+4,+6,+14,+17,+18,+19,+21	X,17,18
12	16/M	6.1	common	54,XY,+X,dup(1),+4,+10,del(13),+14,t(14;16),+ 17,+18,+21,+21	X,4,17
13	16/F	2.2	common	56-60,XX,+X,+4,+6,+10,+11,+14,+18,+21, +22,+mar	X,4,6
14^	3/F	16.8	pre-B	55,XX,+X,+6,+10,+10,+14,+18,+21,+del(14) (q11,q22),+del(18)(q12)	X,6,18
15^	4/M	1.0	common	57-60,XY,+X,+2,+4,+6,+10,+11,+12,+14, +18, +18, +21,+21,+mar1,mar2[cp]	X,4,10
16^	4/M	7.1	pre-B	63-66,XY,+X,der(1),der(1),+2,+4,+5,+6,+7, +8,+10, +11,+12,+16,+17,+18,+19,+20, +21,+22[cp]	X,6,18

^ First investigated in relapse

Fig 6.5

Results of FISH on initial investigation (O) at diagnosis cases 1-13 or in relapse (R) (cases 14-16) and on follow up in remission or relapse (R). Figures in boxes show the percentage of positive cells. Sensitivity (dependent on the number of probes used and the number of cells scanned) was 10^{-4} except where indicated as 10^{-3}

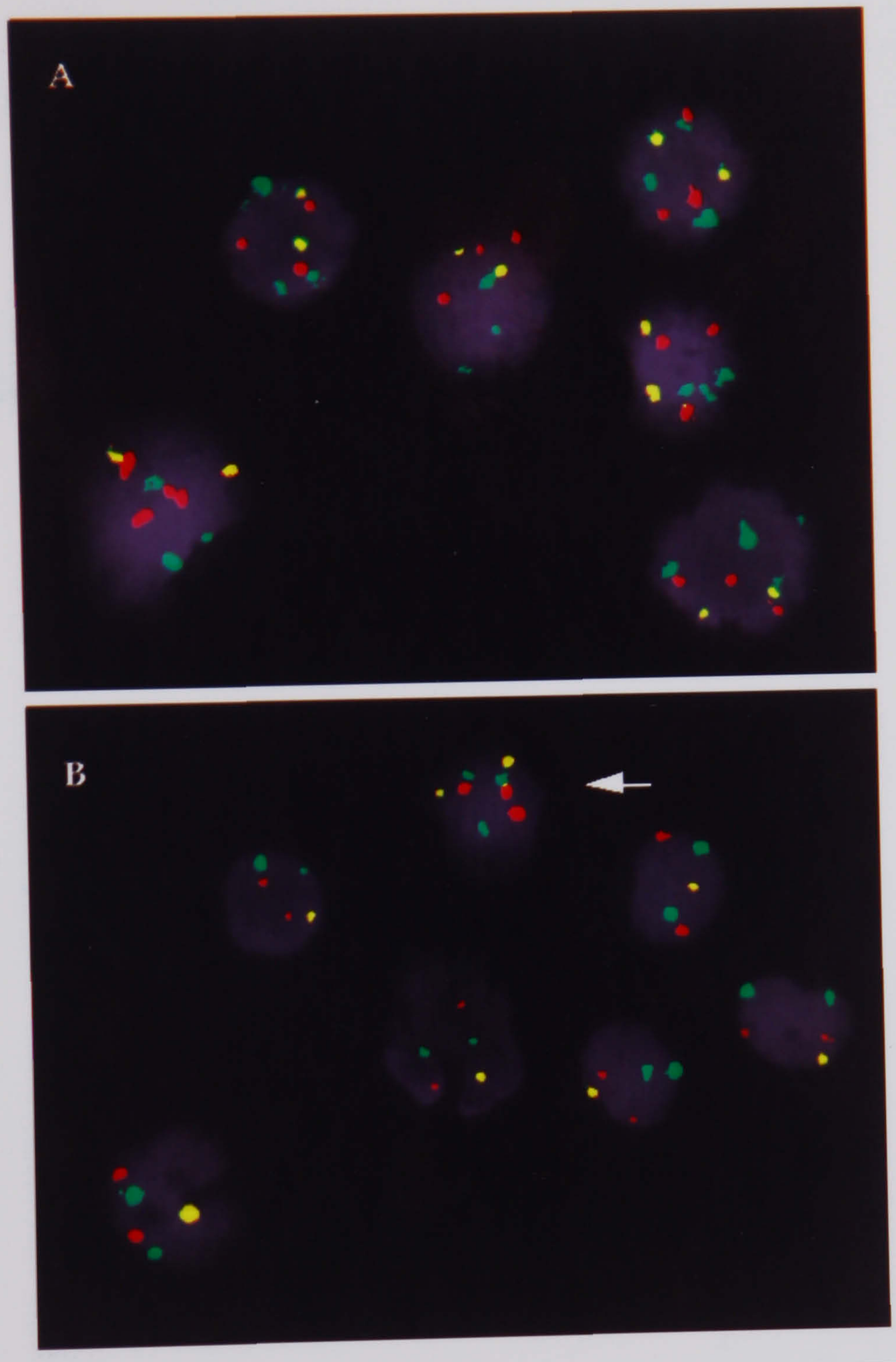


Key

allo BMT = allogeneic bone marrow transplant, R = bone marrow relapse CNS R = central nervous system relapse, figures above boxes = months from initial investigation |> = alive and well.

Fig 6.6

An example of interphase FISH on bone marrow using probes to chromosomes X (yellow), 17 (red), and 18 (green/blue) in a male patient with hyperdiploid ALL. A, at diagnosis, note the majority of nuclei with gains. B in remission, note only one nucleus with gains. These figures are compilations of cells captured separately in order to show all probes in all cells.



6.6 Discussion and conclusions

The study of patients with ALL who had a high hyperdiploid clone at diagnosis or in relapse, in whom minimal residual disease was investigated using an interphase FISH assay is presented. Probes used were to chromosomes known to have been present as trisomies in active disease.

The sensitivity of the interphase FISH assay depends on the level of false positives. The frequency of false positives depends on the particular chromosomal abnormality and the probe or set of probes used for their detection. Others have reported a range of 5%-18% for the detection of monosomy (Kibbelaar et al., 1993; Brizard et al., 1994), 0.5% - 10% for the detection of *BCR-ABL* translocation (Arnoldus et al., 1990; Tkachuk et al., 1990; Chen et al., 1993a; Dewald et al., 1993; Amiel et al., 1995) and 12% for the deletion of the retinoblastoma gene (Stilgenbauer et al., 1993). A range of 0.5%-4% has been reported for the detection of the single trisomies (Rivera et al., 1991; Anastasi et al., 1992; Jenkins et al., 1992; Chen et al., 1993b; Escudier et al., 1993; Kibbelaar et al., 1993; Brizard et al., 1994).

This study has shown that by targetting more than one chromosomal abnormality the rate of false positive cells can be reduced. Using the probabilistic model for the distribution of hybridization signals it was shown that each false gain was an independent event. Targetting two or three chromosomal gains simultaneously has resulted in reducing control values by 100 and 1000 fold respectively. Furthermore the level of sensitivity of the technique has been established by means of a dilution experiment. This showed that the detection of a single gain was limited to one aberrant cell in 10, while dual or triple targetting would detect one cell in 1000 and 10000 respectively. The control values for alpha centromeric probes applied singly in our study were between 1.5% and 2.1% which is similar to that, reported by others (range 2% - 4%) (Anastasi et al., 1992; Jenkins et al., 1992; Escudier et al., 1993; Heerema et al., 1993; Kibbelaar et al., 1993; Brizard et al., 1994). Had control values been below 1%, as reported by some investigators (Chen et al., 1993b; White et al., 1995), our sensitivity experiment using a single probe would have been expected to have enabled detection of one aberrant cell in 100.

The application of the technique benefited from being able to estimate the number of cells in a cytospin from 10 random fields of view. This removed the need for doing a

full count of every cytospin. The availability of an automated scanning system would undoubtedly be of value.

A notable feature of this study was the extremely low level of clonal cells detected in remission samples. These were well below the level of detection which, in this study, could be achieved with a single probe. In contrast, two other studies of MRD in ALL using single-probe FISH have reported persistence of hyperdiploid cells in first remission in 4/12 and 4/20 samples investigated (Heerema et al., 1993; White et al., 1995). All these studies have been small, and follow-up has been short. Differences between studies may be due to chance. It is worth emphasising that while the interpretation of single probe studies is complicated by relatively high control values, this study shows that this is not a problem if a multiprobe approach is used.

FISH for the detection of MRD in hyperdiploid ALL showed heterogeneity between patients in the speed with which the clone was eliminated. The finding that positive samples were most frequent in early remission and that the clone tended to decrease in size with time would be consistent with elimination of the clone with further courses of treatment. Similar results have been reported in studies where PCR has been used for the detection of aberrant fusion products, for immunoglobulin gene rearrangements (Yamada et al., 1990; Nizet et al., 1991; Potter et al., 1993) or T-cell receptor gene rearrangements (Biondi et al., 1992) in remission.

Early elimination of leukaemic blasts from the bone marrow by day 7 (Gaynon et al., 1994), by day 14 (Miller et al., 1994) and by day 28 (Legrand et al., 1994), has been shown to be associated with improved event-free survival. The demonstration of heterogeneity for the persistence of the clone in early remission, albeit at extremely low levels, is worthy of follow-up and raises the possibility that FISH in the early stages may provide important prognostic indicators. In addition regular monitoring of the clone by FISH may, as in one of our cases, enable early detection of a re-emerging clone. This would allow early therapeutic intervention ahead of a full blown relapse.

The use of FISH may have other advantages to patient management in ALL. Examples of these in this study were confirmation of the return of the original clone in a chromosomally normal relapse, similar to that in one of the cases reported by Heerema et al (Heerema et al., 1993). This study demonstrated that the presence of clonal cells at the end of treatment may herald relapse and has shown that central nervous system relapse may be an isolated event. This study confirms two other studies

which using either immunoglobulin gene rearrangements (Yamada et al., 1990) or RT-PCR (Devaraj et al., 1995) have shown lack of bone marrow involvement in a CNS relapse. FISH at diagnosis can also be used to identify chromosomal gain which has been missed due to poor chromosomal morphology.

Until now sensitive methods of MRD detection of chromosomal abnormalities have been limited to the use of RT-PCR for the detection of aberrant fusion products created by certain chromosomal translocations (Potter, 1992; Campana, 1993; Deane, Hoffbrand, 1994; Campana, Pui, 1995). Interphase FISH targetting three chromosomal gains together provides an equivalently sensitive, chromosomally based method of MRD detection for an important subgroup of ALL for which RT-PCR for aberrant fusion gene products is not applicable. This approach to MRD detection is technically simple, leukaemia specific, quantitative and sensitive to 10^{-4}. It may be of advantage to extend its application to any case with a clone showing three concomitant genetic events for which suitable probes are available.

Chapter 7

CONCLUSIONS

The studies carried out and reported in this thesis were designed to gain insight into the size and location of the malignant clone in non-dividing cells from patients with a haematological malignancy. The size of the malignant clone in haematological malignancies at diagnosis and the level of commitment of the target cell for malignant change both determine the extent and severity of the disease and may play an important role in the patient's response to treatment. Persistence of the clone in remission indicates the presence of minimal residual disease (MRD) which may require therapeutic intervention.

The studies described in chapters 3 and 4 concerned samples from patients with haematological malignancies which had been classified cytogenetically as 'failed' or 'normal'. Thus, in these cases, a chromosomally abnormal clone had not been found in metaphase cells. The aim of these studies was to test the hypothesis that chromosomally abnormal clones with hyperdiploidy occur in some of these cases, that the clones are present only in the resting cells and that FISH can be successfully used to detect clones in non-dividing cells. The investigation of sixty five patients with ALL for whom cytogenetic analysis had failed or yielded only normal cells revealed the presence of a hyperdiploid clone in nine cases (chapter 3). The investigation of seven patients with a haematological malignancy and one or two cells with apparently random trisomies at diagnosis or in remission showed the presence of a malignant clone in one case (chapter 4). The presence of a malignant clone in a non-dividing population of cells in a proportion of cases with failed or normal cytogenetics as reported in the studies described above is not surprising. It is well known that clones which are present in less than 11% of the metaphase population are likely to be missed on routine cytogenetic examination and such cases will be classified either as failed or, if at least 20 metaphases can be analysed as chromosomally normal (Hook, 1977). This situation would be expected to occur in cases where the malignant cells are dividing less efficiently in culture than their normal counterparts. As a result a malignant clone which is underrepresented within the dividing population may escape cytogenetic detection. The existence of malignant clones with low survival properties has been described (Manabe et al., 1992). From the study reported in chapter 3 it is clear that both high and low hyperdiploid clones may fail to reach metaphase in short term culture.

The aim of the study described in chapter 5 was to gain insight into the level of commitment of target cell for different cytogenetic abnormalities in the acute leukaemias. It is thought that the striking differences in therapeutic success between different leukaemias can be explained by the differences in the level of commitment of their leukaemic progenitor cells. Thus, most childhood acute lymphoblastic leukaemias and some other paediatric cancers are chemo-curable because they arise in cell populations which are functionally transient, chemosensitive and programmed for apoptosis. In contrast, most adult acute leukaemias are chemo-incurable, at least in part because they originate in relatively drug resistant pluripotent stem cells with extensive self renewal capacity (Jasmin, 1988; Greaves, 1993). The investigation of twelve patients with ALL and five with AML for the clonal involvement of different cell lineages which was carried out in chapter 5 showed that in all cases of AML the abnormality was restricted to the myeloid lineage. In the majority of cases with ALL the clonal abnormality was restricted to the lymphoid lineage. The exceptions were found in the Ph+ subgroup. In this group two cases showed involvement of both lymphoid and myeloid lineages. This study shows that a number of clonal chromosomal abnormalities in acute leukaemia are found only in one type of cell. This suggests that in these cases the acute leukaemia originates in a committed progenitor cell. Another possibility that cannot be ruled out is that the abnormalities might arise in a pluripotent stem cell which is then directed towards either the myeloid (in AML) or the lymphoid (in ALL) lineage only. Philadelphia positive ALL appears to be an exception to this rule. This study showed that in 2/3 cases the Philadelphia chromosome was found in both lymphoid and myeloid lineages, indicative of a pluripotent target cell.

The aim of the study described in chapter 6 was to develop and apply a FISH method for MRD detection of hyperdiploid clones in patients with ALL. The purpose was to provide a method of MRD detection that was as sensitive as PCR in this group of patients. The theoretical analysis showed that false positive hybridization signals that limit the sensitivity of an interphase FISH assay represent probabilistically independent events. Simultaneous targeting of two or more chromosome gains lowered the number of false positive cells and thereby increased the sensitivity of the interphase FISH assay to 10^{-4}. Using this approach a high hyperdiploid clone was successfully detected in remission in a proportion of patients who had a high

hyperdiploid clone at diagnosis. Until now sensitive methods of MRD detection of chromosomal abnormalities have been limited to the use of RT-PCR for the detection of aberrant fusion products created by certain chromosomal translocations (Potter, 1992; Campana, 1993; Deane, Hoffbrand, 1994; Campana, Pui, 1995). The study presented here showed that interphase FISH targetting three chromosomal gains together provides an equally sensitive, chromosomally based method of MRD detection for an important subgroup of patients with ALL for which RT-PCR for aberrant fusion gene products is not applicable.

This thesis demonstrates the value of supplementing metaphase cytogenetics with molecular cytogenetic techniques such as interphase FISH. FISH studies revealed aspects of clonal neoplasia that could not have been discovered by cytogenetic analysis. Furthermore it has been shown that FISH may be used for the detection of certain chromosomal abnormalities in studies of minimal residual disease and this may achieve a high degree of sensitivity.

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Kasprzyk A, Secker-Walker LM (1994) Multiparameter FISH - A Novel Approach for Minimal Residual Disease Detection. Blood 84, Suppl 1:608a(Abstract)

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